出國報告（出國類別：進修）

# 農委會農業菁英培訓計畫
# 雞精子凍存技術發展

服務機關：行政院農業委員會畜產試驗所
姓名職稱：林秀蓮 助理研究員
派赴國家：法國
出國期間：**108**年**9**月**24**日至**112**年**2**月**28**日
報告日期：**112** 年 **5** 月 **1** 日

# 目錄

一、 摘要 ........ 3
二、 目的 ........ 4
三、 研究過程 ........ 5
四、 研究成果 ........ 7
五、 心得及建議 ........ 17
六、 附錄 ........ 19

# 一、摘要

動物遺傳資源保存是維護生物多樣性及確保動物族群在遭受傳染性疾病發生時免於被滅種的有效預防性策略。種原保存包括活體動物繁殖保種與精子、卵子及胚胎凍存等型式。然而基於動物福祉考量，精子凍存目前仍是管理動物遺傳資源最合適的方法之一。冷凍精液具有可長期儲存及遠距離運輸等優點，是種原庫管理及遺傳物質交換不可或缺的要點。此外，優良種公畜禽的遺傳背景更可藉由冷凍精液使其更廣泛的被應用於品種改良。然而，雞冷凍解凍精液進行人工授精的受精率不如新鮮精液，是目前冷凍精液在種雞產業的應用遠不如種畜冷凍精液般普遍的主因。法國國家農業、食品與環境研究院 (INRAE) Nouzilly 中心 ICF 團隊 (Équipe Interactions Cellulaires et Fertilité) 在家畜禽生殖研究領域享譽國際，Dr. Elisabeth Blesbois、Dr. Pascal Mermillod 及 Dr. Anaïs Vitorino Carvalho 分別在家禽精子凍存、精子與輸卵管溝通機制及精子與卵子蛋白質學體領域有卓越的研究成果。執行本計畫三年半期間，先後在三位研究人員的共同指導下學習獲益良多，冷凍雞精液研究之質與能因而往前邁進一大步。

甘油是目前雞冷凍精液最理想的冷凍保護劑，它可提供雞精子優良的保護力以抵抗在冷凍解凍過程的傷害，但其對精子細胞之毒性已被證實，可影響人工授精後的受精率。因此本計畫的第一階段，為探討甘油如何傷害雞精子之構造與功能。試驗結果已確定甘油造成精子活力降低、細胞膜破損、誘發細胞凋亡程序及改變精子能量代謝途徑。接著，依序建立 Hoechst 33342 螢光染色與母雞生殖道精子儲存小管 (sperm storage tubules, SST) 之 in vivo 及 in vitro 試驗模式，以探討甘油化精子的運輸與儲存。由以上的試驗結果得知，甘油化精子由於其構造及功能的改變，因而無法通過母雞陰道 (vagina) 篩選，被拒絕進入位於子宮陰道交接處 (utero-vaginal junction, UVJ) 之 SST 儲存，而造成母雞不孕。

上述結果證實在進行人工授精前移除雞冷凍精液中的甘油是必要的，因此本計畫的第二階段為開發簡單、快速及有效的甘油移除方法，以提高雞冷凍精液的利用價值。為此，我們成功開發 Percoll 膠體離心技術，有效移除雞冷凍精液中的甘油，其平均受精率可達 50%以上。與傳統連續稀釋離心甘油移除法相比，除可節省約 44%的操作時間，並同時簡化設備需求。

事實上，甘油廣泛使用於大部分的家畜禽精子抗凍稀釋液中，而甘油毒性問題也並非僅發生在雞精子上。因此，本計畫所建立的雞精子與生殖道研究模式，將來可以擴大應用於所有動物甘油化精子試驗研究。

# 二、 目的

甘油是常見的動物精子冷凍保護劑，但其對精子細胞之毒性已被證實，可影響人工授精後的受精率。其中，最明顯的案例發生在雞，凍存雞精子的有效甘油濃度為 11%，然而當精液中甘油濃度僅為一半，即 6%時，便會導致母雞完全失去受精能力。由於凍存雞精子操作及使用過程中，主要與甘油共存於兩種不同的溫度：4℃進行冷凍前處理操作，以及 41℃處於授精後輸卵管之生理環境溫度。因此，需探討甘油毒性發生在哪一階段，以及其對精子產生什麼生物功能性傷害。另外，如果甘油確實對精子造成損傷，那麼又該如何防止其對母雞造成不孕的影響也是值得探討的課題。

因此，本研究計畫首先針對甘油在冷凍前與解凍後 4°C，以及授精後 41°C 兩種模擬溫度下對雞精子的生物特性進行評估，包括精子活力、膜完整性、細胞凋亡、粒線體功能以及 ATP 濃度等影響。接著，透過體內授精與體外培養母雞輸卵管精子儲存構造試驗，探討甘油對雞精子在母雞輸卵管中運輸及儲存所造成的影響。研究結果顯示甘油對精子生物特性具有顯著影響，並證實在進行人工授精前移除冷凍解凍精液中的甘油是必要的步驟。為此，我們開發了一種新的方法，利用 Percoll 膠體離心技術，能夠有效地去除雞精液中的甘油，成功地恢復了母雞輸卵管中的精子儲存能力，並在人工授精後達到令人滿意的受精率。

# 三、研究過程

（一）本計畫執行期間為自 108 年 9 月 24 日至 112 年 2 月 28 日，前後在法國 INRAE ICF 研究團隊學習雞精液凍存研究所需之各項關鍵技術，除為攻讀博士之試驗所需，亦有助於返國後持續投入相關家畜禽生殖科技研究發展。各項關鍵技術包括：

1. 公雞精液負壓試管採集
2. 母雞陰道人工授精
3. 精液冷凍解凍及甘油移除新技術
4. 精子螢光染色及流式細胞儀評估精子受精潛能
5. 精子酵素反應生物學品質測定
6. 母雞輸卵管解剖構造
7. 母雞輸卵管組織體外培養
8. 組織細胞玻片影像分析 (Slide Scanner, QuPath, Fiji Image)
9. 統計分析軟體 (RStudio, GraphPad Prism)
10. 科學文章寫作及文獻引用 (Zotero, Mendeley)

（二） 本計畫之試驗研究共分為三大主軸，包括甘油毒性對雞精子生殖受精潛能之研究、雞冷凍精液甘油移除新方法研發及以母雞精子儲存構造驗證甘油移除新方法之價值。各項研究成果摘要簡述如下：

1. 甘油毒性對雞精子生殖受精潛能之研究

甘油造成精子生育力降低之細胞生理機制仍然未知，因此我們首先研究了甘油分別在兩種模擬溫度：4°C 冷凍前處理過程及 41°C 授精後輸卵管生理溫度之影響。在 4°C 條件下 11%甘油會干擾精子生物功能特徵，而在 41°C 條件下 6%甘油即會顯著損害精子生物特性，尤其是精子活力劇烈降低。各項數據揭示了甘油對精子構造及功能的重要影響，這可能造成授精後母雞輸卵管精子儲存減少，解釋了生育力下降的原因。

2. 雞冷凍/解凍精液甘油移除新方法研發

本計畫所研發之 Percoll 膠體離心新技術，可有效地降低雞冷凍解凍精液之甘油濃度至 2%以下，同時並對精子活力具有篩選作用。與傳統的連續稀釋離心技術相比，Percoll 膠體離心新技術除可節省約 50%的樣品處理時間，並可達成較高的受精率。

3. 以母雞輸卵管精子儲存構造驗證甘油移除新方法之價值

Percoll 膠體離心法比連續稀釋離心法展現更強的甘油移除效果。儘管以不同方法處理樣品後未發現對精子體外品質參數產生差異，但卻觀察到精子在母雞輸卵管精子儲存小管之儲存能力及受精率之顯著差異。本研究結果證明雞冷凍解凍精液以 Percoll 膠體離心技術移除甘油之精子，在母雞生殖道有較佳的儲存特性，並反映出較高的受精率及較長的受精持續性。

# 四、 研究成果

（一）本計畫執行至今，發表 SCI 學術期刊二篇 (第一、第二作者各一篇) 及參與多場國際學術研討會進行口頭及海報論文發表。另有三篇學術論文撰寫中，預計於 112 年 12 月前將全數完成投稿 SCI 學術期刊。後續工作包括完成博士論文撰寫，並預計於 112 年 9 月進行博士論文口試。已發表之研究成果及其中文摘要詳述如下：

1. **First insights on seminal extracellular vesicles in chickens of contrasted fertility**
   Reproduction 161(5):489-498 (May 2021)（附錄一）

2. **25ème Journée Thésards de l'Unité PRC**
   Mini-poster and oral presentation (16-17 June 2021)（附錄二）

3. **Chicken semen cryopreservation: importance of cryoprotectants**
   World's Poultry Science Journal 78(1):139-160 (November 2021)（附錄三）

4. **Les 14èmes Journées de la Recherche Avicole et Palmipèdes à Foie Gras**
   Full paper and poster (9-10 March 2022)（附錄四）

5. **Les 30 ans de la Fête de la science**
   Video and poster (2-3 October 2022)（附錄五）

6. **26ème Journée Thésards de l'Unité PRC**
   Mini-poster and oral presentation (15-16 June 2022)（附錄六）

7. **19th International Congress on Animal Reproduction**
   Abstract and poster (26-30 June 2022)（附錄七）

8. **26th World's Poultry Congress**
   Abstract and oral presentation (7-11 August 2022)（附錄八）

9. **Avian Research Symposium 2023**
   Abstract and oral presentation (7-9 March 2023)（附錄九）

## 1. Reproduction 161(5):489-498

**First insights on seminal extracellular vesicles in chickens of contrasted fertility**

**生殖能力優、劣雞隻精液中細胞外囊泡差異的初步研究**

雄性生殖能力低下的原因有多種，其中一些可能與睪丸成熟障礙有關，而這種障礙可能會影響精液成分。在哺乳動物中，細胞外囊泡在與精子進行關鍵交流方面扮演著重要角色，但是在鳥類精液中是否存在細胞外囊泡仍然存有爭議。本研究的目的首先為釐清雞精液中可能存在的細胞外囊泡，其次為證明在具有不同生殖能力的動物中，其細胞外囊泡之大小及蛋白質組成之差異，最後對其與精子的相互作用進行初步評估。

本研究成功地利用超高速離心技術從具有不同精子品質和生殖能力的公雞精液中分離出細胞外囊泡。透過電子顯微鏡觀察，發現雞精液中存在大量的小型細胞外囊泡（可能是外泌體）。比較生殖能力不同的公雞，發現具有較高比例的小型細胞外囊泡，其平均直徑分別為 65.12 和 77.18 奈米。透過 western blotting 發現，不同的細胞外囊泡蛋白質組成不同。在這些標記之中，HSP90A 在生殖能力優良的雄性動物中的豐富程度明顯高於無生殖能力的雄性動物。在共同培養實驗中，生殖能力優良的公雞精液細胞外囊泡顯著影響了精子的存活率以及運動能力。

總體來說，本研究成功地證明了雞精液中存在細胞外囊泡，其大小及蛋白質組成隨著公雞生殖能力狀態的不同而有所差異。這些結果為我們進一步解釋了精液成分對動物生殖能力的影響並提供了重要的訊息，同時也為進一步研究精液細胞外囊泡在生殖醫學和動物繁殖方面的應用提供了基礎。然而，仍需要進一步的研究來了解細胞外囊泡與精子之間的相互作用和其在生殖過程中的作用機制。

（原文見附錄一）

## 2. 25ème Journée Thésards de l’Unité PRC

## New advances in chicken sperm cryopreservation-the issue of glycerol

## 雞精液凍存新進展：解決甘油造成的不孕問題

目前，甘油是冷凍保存雞精液最常用的保護劑，其標準濃度為 11%。然而，在授精前必須除去甘油，以避免其避孕效果。甘油對母雞之避孕機制尚不清楚，為了解決冷凍保存雞精液的挑戰，因而我們進一步研究了甘油對雞精子的細胞生物影響機制，包括細胞膜完整性、粒線體功能、細胞凋亡、ATP 產能、氧化作用等精子生理現象。

除此之外，我們還開發了一種新型甘油去除技術：Percoll 膠體單層離心方法，以便有效應用冷凍解凍雞精液。這種方法成功高效率去除甘油，且相較於傳統方法（連續稀釋後離心），可節省 44%的時間。此外，使用這種方法還能夠有效篩擇具有更高運動性的精子，這是與生育力強烈相關的參數。然而，我們還需要進一步的受精測試，以確保這種方法的實際應用效果。

（原文見附錄二）

## 3. World’s Poultry Science Journal 78(1):139-160

## Chicken semen cryopreservation: importance of cryoprotectants

## 雞精液冷凍保存：冷凍保護劑的重要性

動物遺傳資源的保存是維持生物多樣性的重要策略之外，亦是快速傳播表型性能優良動物基因組成，快速繁殖其後代之有效方法。在活體動物保存基因是可行的方式，但無法避免疾病傳播的風險，此外並需要支付昂貴的動物飼養維護費用。種原冷凍保存是有效的替代策略，除可克服這些缺點，並具有長期儲存、遠距離和低成本運輸以及種原庫空間有效運用管理等優點。相異於哺乳動物，現有技術無法在鳥類物種中保存卵母細胞或胚胎，但可以通過冷凍保存精子、始基生殖細胞和性腺組織來達到相同的目的。而在這些方法中，精子冷凍保存是最實用的方法，因為它具有高效的樣本採集方式、簡單的後代繁殖操作、尊重動物福利的非侵入性操作以及相對高的成功率。

從精液收集開始，雞精子經歷了一系列體外冒險，以完成其生物學使命：受精。精子在整個冷凍保存過程中必須面對並克服幾個關鍵障礙，包括冷凍前處理、冷凍、解凍和人工授精。 此外，有必要在正式採集精液前對個體公雞之精子品質進行評估，以確保其解凍後的受精潛能。日常管理和操作熟練程度等工作項目，決定了新鮮精液的品質。而在進入冷凍前處理階段後，必須做出更多的決定來完成製作冷凍精液，包括稀釋方式、冷凍保護劑 (cryoprotectant agent, CPA) 的選擇、儲存工具的類型、降溫速度和解凍方式等。

甘油是第一個被發現能成功冷凍保存雞精子並繁殖小雞後代的 CPA，然而，甘油具有避孕效果，與雌性動物受精失敗有關，這是 1950 年代以來便發現的歷史性問題，尤其是在雞中。11%的甘油是冷凍雞精子的標準濃度，因為此條件可提供最佳保護力，使精子免受冷凍損傷。然而，授精時精液中僅存在 2%的甘油就足以誘導生育能力下降，而 6%的甘油更是會導致母雞完全不育。到目前為止，我們僅部分了解甘油對精子運動、呼吸代謝和頭帽反應等生物學特性產生影響，也知道它可能會改變母雞生殖道中的精子儲存和蛋白質分泌。儘管在過去的 70 年裡一直在討論甘油造成不孕之議題，但目前僅有非常有限的訊息可以解釋為什麼它對精子的受精能力如此有害。

善用甘油出色的冷凍保護能力或因其毒性而放棄使用在抗凍稀釋液中，是一個兩難的選擇。儘管如此，甘油目前仍然是雞精子冷凍保存中最常用的 CPA。為了避免其毒性

干擾生殖能力，在雞冷凍精液使用上，已開發出一種稱為連續稀釋離心的技術，專門用於在授精前去除甘油，實現了可接受的受精率。然而這項技術涉及特定的設備、複雜的工作程序和耗費時時間等缺點，因而有必要開發一種簡單、快速和靈活的方法來提高雞冷凍-解凍精液的利用效率。此外，必須更深入地了解甘油毒性和受精失敗的細胞生理和分子機制，期將來可獲得一種新穎且更有效控制甘油毒性的實用策略。

（原文見附錄三）

## 4. Les 14èmes Journées de la Recherche Avicole et Palmipèdes à Foie Gras

**Élimination du glycérol de la semence décongelée de coq par centrifugation dans un gel colloïdal synthétique**

利用合成膠體離心分離法去除雞冷凍解凍精液中的甘油

精液冷凍保存是保護物種和品種生物多樣性、在緊急傳染病中恢復動物族群的重要方法。目前，甘油被廣泛用作冷凍保護劑，可以有效維持精子功能。然而，甘油卻會導致人工授精時的受孕能力降低或完全不孕，因此研究人員嘗試開發一種簡單的方法，使用合成膠體 Percoll 透過離心方式，以去除雞精液中之甘油。在收集雞精液後，將其與 11％甘油的稀釋劑混合，裝入程式降溫冰箱中進行冷凍。解凍的精液被分層在含有 200 mM 蔗糖和 Percoll 的 PBS 中進行 15 分鐘離心。結果顯示，所有濃度的 Percoll 膠體配方都可以有效地將甘油濃度降低至 120 mM 以下，相當於從解凍精液中去除 94％的甘油。此外，此方法可以在 20°C 下進行操作，並可以有效地選擇運動和前進性較好的精子，特別是使用 Percoll 40 和 70％。該研究顯示，這種新方法可以有效地從雞精液中去除甘油，同時積極地選擇運動性較好的精子。與傳統的連續稀釋方法相比，使用該方法可以節省 44％的甘油移除操作時間。

（原文見附錄四）

## 6. 26ème Journée Thésards de l’Unité PRC

Exploring how glycerol impacts fertility capacity of chicken sperm

探究甘油對雞精子生育能力的影響

甘油對於脊椎動物精子的保護效果非常出色，能夠減少因低溫冷凍引起的損傷。在雞冷凍解凍精液的應用上，11%的甘油濃度是雞冷凍精液的標準濃度，但在人工授精前需要將甘油去除，以避免產生不孕作用。這個現象自 1950 年代以來廣受討論，但對於甘油對生殖效果產生負面影響的機制仍不明確。因此，本研究假設甘油會在授精前 (在冷凍和解凍過程中) 和授精後 (在雌性生殖道中) 干擾精子的細胞生物功能。首先，我們確認了不同甘油濃度對精子生育能力的影響,然後探究了精子在 4°C 和 41°C 下與甘油共存 60 分鐘 (此為精子在授精後進入母雞生殖道，前進至精子儲存或受精部位所需的時間) 的影響。

在 41°C 下，甘油對精子運動能力和生物功能產生明顯的影響，可能因此改變了精子在母雞生殖道中前進、篩選和儲存的正常功能，從而導致母雞生育能力下降。ROS 平衡和 ATP 含量是調節精子運動能力的兩個主要因素,因此未來的體外研究將進一步驗證它們之間的聯繫來解密甘油化精子在母雞生殖道中產生的有害影響。

（原文見附錄六）

## 7. 19th International Congress on Animal Reproduction

## Impact of glycerol on sperm fertilizing capacity in chicken

## 甘油對雞精子受精能力的影響

甘油是脊椎動物精液最常用的抗凍保護劑，因為它能夠有效地保護精子免受低溫造成的細胞傷害。在雞中，11%的甘油是冷凍精液的標準濃度，但必須在母雞授精之前去除，否則將大幅降低其受精能力。這種現象自 1950 年代以來就被討論，但其涉及的機制仍不清楚。在本研究中，我們假設甘油可以在低溫下保留精子生物特性，但在生理溫度 41°C 下會干擾精子生物功能。因此，我們首先研究增加甘油濃度對母雞受精能力的影響。接著，我們探討了甘油存在於 41°C 下 60 分鐘內 (即精子從陰道到漏斗授精部位的生理時間)，如何損害精子生物特性，特別是精子運動性和細胞膜完整性。本試驗收集了 10 隻成年 T44 公雞的精液，混合後以甘油-Lake PC 稀釋液在 4°C 下稀釋至甘油最終濃度 0、1、2、6 和 11％。對於受精能力測試，每隻母雞授精 $100\times10^6$ 甘油化精子，在稀釋後立即進行精液注射。對於體外實驗，於 41°C 下 0、10、20 和 30 分鐘使用顯微鏡和計算機輔助精子分析系統 (CASA) 分別評估精子群體和個體運動性，並在 0、30 和 60 分鐘時使用螢光流式細胞儀評估精子膜完整性。結果顯示，2%的甘油導致受孕率下降了 50%，而使用 6%和 11%的甘油會完全導致母雞完全不育。甘油對精子的群體運動性沒有影響，但在培養 10 分鐘後，1%的甘油明顯降低了精子的個體運動性，並且更高濃度的甘油會導致更嚴重的降低。此外，雖然 1%和 2%的甘油對精子沒有影響，但 6%和 11%的甘油在 41°C 培養 30 分鐘後會導致精子失去膜完整性。綜上所述，即使是極低濃度的甘油 (1%) 存在於精液樣本中也會影響母雞受精能力和精子運動性，而更高的濃度也與膜缺陷有關，這證實了在甘油化精子注射前需要去除甘油以維持精子完整性及其在母雞生殖道中的正常運輸與儲存。

（原文見附錄七）

## 8. 26th World’s Poultry Congress19th

## Application of colloidal centrifugation to remove glycerol from chicken frozen-thawed semen

## 利用膠體離心技術去除雞冷凍-解凍精液中的甘油

甘油在冷凍-解凍過程中可提供細胞卓越的保護作用，是最常使用的精子抗凍保護劑。然而，雞精液中存在甘油會導致嚴重的受精失敗，因此必須在授精之前將其去除。因應這個問題，在使用雞冷凍-解凍精液已開發出一種特殊方法來去除甘油，即連續稀釋離心技術 (SD)，然而其需要特殊設備將解凍的精液保持在 4°C，且操作耗時。因此，我們開發了一種簡單的方法，使用膠體 Percoll (通常用於哺乳動物及新鮮雞精液中篩選高活力精子) 進行冷凍雞精液在解凍過程中的甘油去除。18 隻 T44 成年公雞隨機分為三組用作採集精液試驗動物，然後在程式降溫儀中冷凍含 11%甘油-Lake PC 稀釋液麥管。將冷凍精液在 4°C 下解凍 3 分鐘，放置在含有 200 mM 蔗糖和等張 Percoll (Sucrose-Percoll solution, SPS) 的 PBS 溶液上，在 20°C 下 800 × g 離心 15 分鐘。為測試了不同濃度的 SPS-PBS (40、50、60 或 70%) 以識別最佳操作條件，因而進行精子品質體外試驗，包括甘油濃度測定和精子個別運動性評估作為體內受精能力的指標。接著使用經 SPS 和 SD 方法移除甘油的冷凍-解凍精液進行母雞人工授精，以比較甘油移除後之精子受精能力。綜合而言，我們新開發的 SPS 膠體離心法可以有效地從雞冷凍-解凍精液中去除甘油，同時對精子運動性進行正向篩擇。雖然 SPS 40％的受精能力比 SPS 70％高，但 SPS 70％與 SD 處理後的受精能力沒有顯著差異。與傳統的 SD 方法相比，SPS 新技術可以節省 44％的操作時間，同時提升母雞受精率，歸納出這種新技術具有簡單、快速和高效率的優勢，建議用於雞冷凍精液的解凍操作流程中。

（原文見附錄八）

## 9. Avian Research Symposium 2023

### Impacts of glycerol on sperm fertilizing ability in chickens

### 甘油對雞精子受精能力的影響

甘油是冷凍雞精子最有效的保護劑，但其會導致母雞受精失敗，需要在授精前去除。這些造成生育能力降低的細胞作用機制仍不清楚。本研究首先探討了甘油 (0、1、2、6和 11%) 對雞精子在兩種溫度下 (4°C 模擬冷凍及解凍過程和 41°C 模擬母雞輸卵管生理溫度) 培養 60 分鐘對精子生物學的影響。收集 10 隻 T44 公雞精液，混合後與不同劑量的甘油共同培養，評估精子運動性、膜完整性、細胞凋亡、粒線體活性和 ATP 濃度等參數。其次，我們探討甘油如何影響授精後精子在母雞輸卵管的運輸和儲存。將 Hoechst 螢光染色的精子進行 9 隻母雞陰道人工授精 ($200 \times 10^6$ 精子/母雞)，24 小時後犧牲母雞並分離輸卵管，接著在陰道子宮交界處探測精子儲存小管中精子的存在。結果顯示，11%的甘油在 4°C 下是唯一對精子生物學產生干擾的濃度，而 6%和 11%的甘油在 41°C 下會顯著影響精子生物特性，尤其是精子活力。此外，隨著甘油濃度的增加，精子儲存小管中的精子數量也減少。綜合以上數據揭示了甘油對精子活力的重要影響，這可能是導致授精後輸卵管精子儲存小管中精子減少的原因，解釋了母雞生育能力降低的現象。（原文見附錄九）

# 五、 心得及建議

1. 在法國國家農業、食品與環境研究院 (INRAE) 攻讀博士期間，我進行了多項研究，包括雞冷凍精液的製備、解凍精液的配種，以及母雞輸卵管精子儲存組織的培養等實驗。在這些動物試驗中，必須獲得各項技術的支持與協助，包括公雞精液採集、母雞人工授精、母雞解剖輸卵管分離和受精蛋孵化等。INRAE 的家禽飼養單位 (PEAT) 專門提供試驗動物以供研究人員使用，研究人員可以透過研究計畫經費支付動物使用費用，以訂購所需特殊動物品種/品系，並在專業固定的飼養條件下，取得品質穩定的試驗動物，同時與 PEAT 技術純熟的動物飼養人員協同進行動物試驗操作。在此動物供應模式下，研究人員得以節省大量時間，專注於樣品操作、數據分析和研究報告撰寫，進而提升研究效率。

2. 在 INRAE 的組織架構中，所有的精密儀器，例如組織細胞玻片掃描儀和共軛焦顯微鏡，以及試驗所需的相關軟體，例如影像分析軟體和統計分析軟體，皆有專責的管理單位和具有專業知識的操作人員負責。因此，當研究人員有需要時，可以即時獲得技術支援。

3. 已知成熟精細胞之蛋白質轉錄和翻譯能力非常有限，因此已存在精細胞或精漿之蛋白質被認為是精細胞表現的主要功能性物質。精子蛋白質體學 (proteomics) 有助於了解配子發生作用 (gametogenesis) 的細胞生理途徑、轉譯後修飾和蛋白質–蛋白質相互作用以及蛋白質在受精過程中扮演的關鍵功能。此外，精子蛋白質體學更有助於建立雄性生殖指標的生物標記 (biomarkers)，除可用於家畜禽配種繁殖參考，亦可用於預測人類醫學以及瀕危品種和物種的遺傳保種計畫。蛋白質體學研究目前已被廣泛應用於動物繁殖生理研究，若本所可順利建立相關研究技術，預期可開啟多項家畜禽繁殖研究計畫，包括可應用於優良種畜禽選拔配種研究及冷凍精液、卵子、胚胎及體外受精 (in vitro fertilization, IVF) 等各項人工生殖技術研發。

4. 類器官（organoid）是由多種細胞組成的 3D 立體結構，能夠通過體外的 3D 組織培養技術模擬器官在體內的生長環境。相較於傳統的 2D 細胞培養方法，3D 組織培養技術更能夠模擬細胞與細胞之間、細胞與細胞外基質之間的複雜交互作用。近年來，

隨著研究人員對器官的細胞生理功能及調控機制更深入的瞭解，越來越多的研究開始使用類器官培養技術作為重要的研究工具。在這個領域中，INRAE ICF 團隊的 Dr. Pascal Mermillod 和 Dr. Karine Reynaud 在家畜禽類器官培養的研究方面具有傑出的研究成果。

5. 目前已與法國 INRAE 的指導教授 (Dr. Pascal Mermillod 和 Dr. Anaïs Vitorino Carvalho) 針對精子蛋白質體學及類器官培養方面的未來研究合作，進行初步討論與籌劃。我們期望能建立畜產試驗所與法國 INRAE 之間持續緊密的長期合作關係，攜手探究繁殖生理研究領域中的重要問題，共同推進該領域的前沿發展。

# 六、 附錄

附錄一

REPRODUCTION
RESEARCH

# First insights on seminal extracellular vesicles in chickens of contrasted fertility

Luiz Cordeiro[1], Hsiu-Lien Herbie Lin[1,2], Anaïs Vitorino Carvalho[1], Isabelle Grasseau[1], Rustem Uzbekov[3] and Elisabeth Blesbois[1]

[1]INRAE, CNRS, Université de Tours, IFCE, UMR PRC, Nouzilly, Centre-Val de Loire, France, [2]Physiology Division, Livestock Research Institute, COA Tainan, Taiwan and [3]Laboratoire Biologie Cellulaire et Microscopie Electronique, Fac. Médecine, Université de Tours, Tours, Centre-Val de Loire, France

*Correspondence should be addressed to E Besbois; Email:* elisabeth.blesbois@inrae.fr

## Abstract

**Male subfertility causes are very varied and sometimes related to post-gonadic maturation disruption, involving seminal plasma constituents. Among them, extracellular vesicles are involved in key exchanges with sperm in mammals. However, in birds, the existence of seminal extracellular vesicles is still debated. The aim of the present work was first to clarify the putative presence of extracellular vesicles in the seminal plasma of chickens, secondly to characterize their size and protein markers in animals showing different fertility, and finally to make preliminary evaluations of their interactions with sperm. We successfully isolated extracellular vesicles from seminal plasma of males showing the highest differences in semen quality and fertility by using ultracentrifugation protocol (pool of 3 ejaculates/rooster, *n*=3/condition). Size characterization performed by electron microscopy revealed a high proportion of small extracellular vesicles (probably exosomes) in chicken seminal plasma. Smaller extracellular vesicles appeared more abundant in fertile than in subfertile roosters, with a mean diameter of 65.12 and 77.18 nm, respectively. Different protein markers of extracellular vesicles were found by western blotting (*n* = 6/condition). Among them, HSP90A was significantly more abundant in fertile than in subfertile males. In co-incubation experiments (*n* = 3/condition), extracellular vesicles enriched seminal fractions of fertile males showed a higher capacity to be incorporated into fertile than into subfertile sperm. Sperm viability and motility were impacted by the presence of extracellular vesicles from fertile males. In conclusion, we successfully demonstrated the presence of extracellular vesicles in chicken seminal plasma, with differential size, protein markers and putative incorporation capacity according to male fertility status.**

*Reproduction* (2021) **161** 489–498

## Introduction

Seminal plasma, the fluid part of semen, is a highly complex fluid originating from secretions of the male tract. It interacts actively with sperm, and plays a key role in sperm maturation and fertilizing ability (Maxwell *et al.* 2007, Rodríguez-Martínez *et al.* 2011, Druart & de Graaf 2018). The composition and functions of seminal plasma differ greatly between species. For example, due to the lack of specific secretion glands such as prostate or seminal vesicles in the male tract, the seminal plasma of birds is very different from the mammalian seminal plasma, and consequently, the post-gonadic maturation process also (reviewed by Blesbois & Brillard 2007, Blesbois 2012, Santiago-Moreno & Blesbois 2020). The various investigations performed in mammals revealed that extracellular vesicles (EVs) present in seminal plasma are involved in the post-gonadic sperm maturation (Ronquist & Brody 1985, Sahlén *et al.* 2002, Saez *et al.* 2003, Sullivan *et al.* 2005, Frenette *et al.* 2010).

EVs are membrane vesicles involved in the transfer of proteins, lipids and nucleic acids between two cells, as signaling vehicles to maintain the normal cell homeostasis or respond to pathological events (Van Niel *et al.* 2018). There are two main categories of EVs defined from their size and biogenesis process: the exosomes (30–150 nm mean diameter) produced from endosomal system and the microvesicles (50–500 nm mean diameter) derived from the plasma membrane (Raposo & Stoorvogel 2013, Van Niel *et al.* 2018). However, mainly due to the overlapping range of size and the similar morphology, the definition of the proportion of sub-species of EVs remains a true challenge from the current available protocols of isolation (Van Niel *et al.* 2018). EVs formation involves specific proteins to regulate their biogenesis (e.g. programmed cell death 6-interacting protein – PDCD6IP), to maintain protein folding and membrane fusion (chaperones including Heat Shock Protein 90 kDa alpha – HSP90A, Valosin-containing protein-VCP), to regulate EVs-fusion with the target cells

© 2021 Society for Reproduction and Fertility
ISSN 1470–1626 (paper) 1741–7899 (online)

https://doi.org/10.1530/REP-20-0462
Online version via https://rep.bioscientifica.com
Downloaded from Bioscientifica.com at 04/09/2022 07:56:17AM
via free access

(annexins like ANXA5) (Buschow *et al.* 2010, Théry *et al.* 2018, Van Niel *et al.* 2018, Munuce *et al.* 2019).

Seminal EVs are secreted by different part of the mammalian male reproductive tract. They are generally named after the production localization, such as epididymosomes if secreted by the epididymis (Frenette *et al.* 2002, Saez *et al.* 2003, Frenette *et al.* 2010, Chen *et al.* 2016), or prostasomes if produced by the prostate (Renneberg *et al.* 1997, Sahlén *et al.* 2002, Sullivan *et al.* 2005, Burden *et al.* 2006, Ronquist 2012). Seminal EVs have multiple impacts on sperm biology including sperm maturation (Sullivan *et al.* 2005) especially by modifying sperm motility (Fabiani *et al.* 1994, Arienti *et al.* 2004), plasma membrane integrity (Du *et al.* 2016), and capacity to acrosome reaction (Murdica *et al.* 2019). They are also involved in the embryonic development (Conine *et al.* 2018) and may be sufficient to transfer a paternal phenotype to offspring (Chan *et al.* 2020).

Due to their importance in sperm biology, numerous authors suggest that the EVs may be markers of in/subfertility in mammals (Gabrielsen & Lipshultz 2019, Vickram *et al.* 2020). Despite no difference of shape or size, Murdica *et al.* (2019) revealed a difference of action of EVs on sperm biology (motility and acrosome reaction) depending on the patient fertility status. Furthermore, recent proteomic investigations revealed a list of more than 90 proteins differentially abundant between EVs isolated from seminal plasma of normozoospermic and asthenozoospermic patients (Lin *et al.* 2019). Collectively, these data suggest that EVs contents modulate sperm fertilizing ability. Despite the presence of EVs protein markers in rooster seminal plasma (Labas *et al.* 2015, Borziak *et al.* 2016, Li *et al.* 2020), the presence of EVs could not be described (Alvarez-Rodriguez *et al.* 2020). Consequently, the EVs existence in the seminal plasma of roosters is still debating and their potential link to the animal fertility status remains unexplored.

In this context, the first aim of the present study was to isolate rooster seminal plasma EVs. Then, we evaluated size and protein markers according to the fertility ability of the animals. Finally, we also investigated the interaction of enriched fractions of seminal EVs with sperm activity.

## Material and methods

### *Animals*

All experiments were carried out in accordance with the legislation governing the ethical treatment of birds and were approved by the French Ministry of Higher Education, Research and Innovation, and the Val-de-Loire Animal Ethics Committee (authorization no. DAP: APAFIS#4026-2016021015509521). On the 40 adult T44 roosters (*Gallus* gallus domesticus, Sasso, France), 39 were semen donors. 80 ISA Brown adult hens were used for fertility tests. All roosters were breeding into individual cages and the hens reared in cages of four animals, housed at the INRAE experimental unit UE-PEAT INRAE Poultry Experimental Facility (2018, https://doi.org/10.15454/1.5572326250887292E12). All the animals were 35–40 weeks old. They were maintained under a lighting regimen 14 h light:10 h darkness cycle, controlled temperature at 21°C, feeding with a standard diet and water *ad libitum*.

### *Semen collection*

Semen from each rooster was collected individually twice a week (2–3 days between two collections) in 200 µL of a specific collection extender according to experiment (see subsequently), by dorso-abdominal massage as previously described (Burrows & Quinn 1937). Care was taken to avoid any contamination with transparent fluid and other cloacal products.

On the 40 tested animals, only one was infertile (absence of ejaculate) and was not used in this investigation.

### *In vitro semen evaluation and fertility test*

Semen of the 39 males was collected in Beltsville poultry semen extender (BPSE) (Sexton 1977). Sperm volume was defined by weighing (mg) and sperm concentration was determined by light absorption using a photometer (Accucell photometer, IMV Technologies, L'Aigle, France) at a wavelength of 540 nm (Brillard & McDaniel 1985). Sperm viability (%) was determined with SYBR-14/Propidium iodide fluorescent dyes (Molecular Probes™ LIVE/DEAD™ SYBR-14/propidium iodide fluorescent dyes, L7011, Invitrogen, France), and revealed by flow cytometry (EasyCyte Guava, IMV Technologies, France) (Thélie *et al.* 2019). The proportion of live and dead sperm was assessed on a total of 5000 sperm per sample. Mass motility (movement of sperm group) was defined on a motility scale previously described (Blesbois *et al.* 2008): zero as a total lack of movement and nine as the presence of representing whirlwinds covering 30–60% of the observed area. Objective measurements of percentage of motile sperm (%) were evaluated by the computer-assisted sperm analysis (CASA) system with an HTM-IVOS (Hamilton-Thorn Motility Analyzer, IVOS, IMV Technologies), as previously described (Nguyen *et al.* 2014). The percentage of motile sperm was retained as the most robust parameter for further analyses.

A dose of $100 \times 10^6$ sperm/female was used for intravaginal inseminations of 8 hens per male (females laying rate higher than 95%). The eggs were collected daily and put into incubation. The fertility rate (number fertile eggs/number incubated eggs × 100) was estimated after 8 days of incubation by candling the eggs laid between days 2 and 8 after a single insemination. The highly fertile group (F) was defined by fertility > 90% and the subfertile group (SF) by fertility <45%. The experimental candidates selected for the EVs enriched fractions were selected according to their fertility level (F > 90%, SF < 45%) and also percentage of motile sperm (F > 50%, SF < 30%) and mass motility (F ≥ 6.5, SF < 3) differentials.

### *Seminal extracellular vesicles isolation*

For this specific preparation, semen was collected individually (with a minimum interval of 3 days between collects)





Downloaded from Bioscientifica.com at 04/08/2023 07:56:17AM
via free access

in PBS (Sigma) added with protease inhibitor EDTA-free (04693159001, Roche Diagnostics). The first seminal plasma fraction (clarification) was obtained individually from total semen centrifugation at 959 ***g*** (Centrifuge 5417R, Eppendorf, France) for 10 min at 4°C. The supernatant, clarified seminal plasma, was collected and stored in ice. Sperm pellet was gently resuspended with 500 µL PBS with protease inhibitor and centrifuged as performed before. Supernatant were collected and added to clarified seminal plasma. Pellet was resuspended a second time in 500 µL PBS with protease inhibitor and placed on agitation 500 rpm (Thermomixer compact, Eppendorf, France), 10 min at 37°C. A third centrifugation was performed as described before and the supernatant was pooled with the previous supernatant fractions. The total volume of seminal plasma was centrifuged at 15,294 ***g*** for 15 min at 4°C. The supernatant was collected containing seminal plasma without cellular debris and stored at −20°C for further EVs isolation.

EVs isolation was carried out on resulting purified seminal plasma (pool of three collections per animal per replicate). EVs isolation was performed by two successive ultracentrifugations at 100,000 ***g*** for 90 min at 4°C (Beckman model L8-M with SW-55-Ti rotor, adjusted k-factor: 163, using the formula $k = (2.533 \times 10^{11}) \times \ln(rmax/rmin)/rpm^2$, were r(min) = 60.8mm and r(max) = 108.5mm at 30,000 rpm), with a washing step with PBS with protease inhibitor (Théry *et al.* 2006, Doyle & Wang 2019). The pellet obtained (EVs-enriched) from the second ultracentrifugation was suspended in 50 µL of PBS with protease inhibitor for transmission electron microscopy observations and western blotting experiments or in 50 µL Lake 7.1 (Lake & Ravie 1981) with protease inhibitor for EVs co-incubation tests with sperm.

### Western blotting analysis

Western blotting experiments were performed for EVs protein markers (Buschow *et al.* 2010, Théry *et al.* 2018, Van Niel *et al.* 2018, Munuce *et al.* 2019) on individual samples of EVs suspension (6 F and 6 SF roosters). In each sample was added 100 µL lysis buffer (150 mM NaCl ,10 mM Tris HCl, 1 mM EGTA, 1 mM EDTA, 200 mM sodium fluoride, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 % Triton X-100 and 0,5 % NP40) with protease inhibitor EDTA-free, sonicated and centrifuged at 12,000 ***g*** during 10 min at 4°C. Supernatant was collected and protein concentration was determined by the DC™ Protein Assay kit (5000112, BioRad, USA) according to the manufacturer's instructions. Samples were diluted in protein loading buffer (928-40004, LI-COR Biosciences, USA) added 10% (v/v) 2-Mercaptoethanol (M-7522, Sigma-Aldrich) and heated at 95°C for 5 min. From diluted samples, 20 µg of proteins were loaded on 10% SDS-PAGE, before being transferred to nitrocellulose membranes (10-600001, Amersham Protran 0.2 µm NC, GE Healthcare Life Science), in triplicate. Full-length protein loaded on gel were measured from images obtained from membranes stained with Revert™ 700 Total Protein Stain (926-11011, LI-COR Biosciences, USA) according to the manufacturer's instructions, using Odyssey CLx Imaging System (LI-COR Biosciences, USA) and quantified using Image Studio™ Lite Software (LI-COR Biosciences, USA). Membranes were washed with TBST (TBS, N14581, Interchim Life Sciences, France; 0.1 % (v/v) Tween-20, P9516, Sigma, Germany), incubated 1 h in the blocking solution (5 % (w/v) non-fat dry milk in TBST) at room temperature, and then incubated with the primary antibody overnight at 4°C in the blocking solution under agitation. The primary antibodies used were against: ANXA5 (36 kDa, 1:1000, rabbit, CSB-PA06384A0RB, Cusabio), and HSP90A (90 kDa, 1:1000, mouse, ab59459, Abcam), VCP (90 kDa, 1:1000, mouse, ab11433, Abcam) and PDCD6IP (96 kDa, 1:500, rabbit, HPA011905, Sigma). After washing, membranes were incubated at room temperature for 2 h with IRDye 800CW goat anti-rabbit (926-32211, LI-COR Biosciences, USA) or IRDye 800CW goat anti-mouse (926-32210, LI-COR Biosciences, USA) diluted 1:2000 in the Odyssey blocking buffer (927-50000, LI-COR Biosciences, USA) 1:1 (v/v) in TBS. The image of protein band signals was captured using Odyssey CLx Imaging System and quantified using Image Studio™ Lite Software and relative concentration was calculated using full-length protein quantification on membrane stained with Revert™ 700 to band signal normalization.

### Characterization by transmission electron microscopy

From EVs-enriched suspension, 10 µL aliquot was thawed in ice during 1 h with the same volume of Trump's fixative solution (4% formaldehyde, 1% glutaraldehyde in PBS). The 3 µL aliquot was placed on a Formvar carbon-coated grid for 5 min. Samples were washed with distilled water (three times, 10 s), then for negative contrast stained with 2% uranyl acetate (three times, 10 s) and air dried at room temperature. The electron-micrographs were obtained using TEM HITACHI HT 7700 Elexience at 80 kV (with a charge-coupled device camera AMT) and JEM 1011 (JEOL, Japan) equipped with a Gatan digital camera driven by Digital Micrograph software (Gatan, Pleasanton, USA) at 100 kV. From each sample (3 F and 3 SF), all extracellular vesicles were measured from the 10 field photos using ImageJ software version 1.51n (http://imagej.nih.gov/ij, NIH).

### Co-incubation of sperm with extracellular vesicles

Individual semen from 3 F and 3 SF roosters was collected in tube containing 200 µL Lake 7.1 added with protease inhibitor EDTA-free. Seminal plasma was removed by centrifugation at 600 ***g*** (2-16PK, Sigma), 10 min at 20°C and sperm concentration was determined as previously described. Diluted at $100 \times 10^6$/mL, sperm were incubated with 10 or 50 µg of EVs-enriched suspension pooled from 3 F animals, or without EVs, at 37°C, in water bath. Co-incubations EVs-sperm were proceeded for motility and viability measurements, as previously described, after short periods of co-incubation (5, 10 and 15 min) since the sperm quality decreases very rapidly for longer time at this temperature (Lemoine *et al.* 2008). Experiments were performed in triplicate.

### Extracellular vesicles incorporation by rooster sperm

To evaluate the EVs incorporation by sperm, lipophilic green fluorescence dye PKH67 (MINI67, Sigma) was used to stain the EVs as previously described (Saadeldin *et al.* 2014). EVs-



Downloaded from Bioscientifica.com at 04/08/2023 07:56:17AM
via free access

enriched fraction of 3 F or 3 SF roosters (10 µL EVs-enriched fraction/animal; 30 µL final solution) was stained with PKH67 according to the manufacturer's instructions. Same volume of PBS was stained in parallel and used as negative control. Stained EVs were stored at −20°C, protected from light until co-incubation with sperm.

Semen from the 3 F and 3 SF roosters were prepared as described above in Lake 7.1. The F and SF sperm were co-incubated with stained EVs (10 µg of total protein) originating from F or from SF animals, in 1 mL Lake 7.1, protected from light, at 37°C in water bath during 1 h. One microliter of each incubation was applied in glass pre-treated with poly-L-lysine (VWF International, Germany) and coverslips were applied with Fluoroshield mounting medium with DAPI (Sigma). Individual sperm cells were analyzed using a confocal laser-scanning microscope (LSM700, Zeiss) equipped of excitation wavelengths 488 to 555 nm. All experiments were performed in triplicate.

### Statistical analysis

All statistical analyses were performed with R software version 3.6.3 (R Core Team 2017) using the following packages: nlme (version 3.1-147) (Pinheiro & Bates 2013), lsmeans (version 2.30-0) (Lenth 2016) and multcomp (version 1.4–13) (Piepho 2004). Statistical testing was performed on datasets at a statistical significance of 5%.

#### *In vitro semen quality*

The Student's *t*-test was used for comparison of each parameter of *in vitro* semen evaluation among different groups. $P < 0.05$ was considered statistically significant.

#### *TEM analysis*

Size was determined for 3724 EVs from 3 F roosters and 1660 obtained from 3 SF animals. EVs was classified in three categories: < 70 nm, between 70 and 100 nm, > 70 nm and their distribution in F and SF groups was analyzed by $chi^2$ test.

#### *Protein abundance*

Relative abundances of proteins present in EVs and obtained by western blotting on 6 F and 6 SF roosters were analyzed by Wilcoxon–Mann–Whitney test.

#### *PKH67 relative intensity*

Three animals of each fertility groups were used to investigate PKH67 relative intensity in sperm. First, the impact of the fertility group of EVs and the fertility group of sperm was investigated with a linear model (ANOVA test).

Post-hoc analyses with Tukey's multiple comparisons adjustment were performed depending to the significant terms. Then, the presence and origin of EVs was focused in only F sperm with a linear model (ANOVA test) Post-hoc analyses were performed with Tukey's multiple comparisons adjustment.

#### *Viability and motility in co-incubation experiments*

Based on the data collected from 3 F and 3 SF roosters, the impact of EVs, time and their interaction on sperm viability and motility was estimated following a mixed linear model for modeling heteroscedasticity and correlated errors. Each fertility group and each EVs quantity was analyzed separately. Post-hoc analyses with Tukey's multiple comparisons adjustment were performed depending to the significant terms.

## Results

### Fertility test

The individual fertility rates of the 39 males producing ejaculates ranked from 20 to 96%. Seven representative roosters were used in this work to constitute F samples with an average fertility rate of 95.07 ± 0.90%, means % motile sperm 57.4 ± 4.88%, and mass motility 6.79 ± 0.22. The 6 representative selected SF animals had an average fertility rate of 33.69 ± 2.81% and a mean % motile sperm 23.88% ± 7.05, and mass motility 3.61 ± 0.80. All these parameters were significantly different between groups ($P < 0.002$). Among all the animals analyzed, 7 F and 6 SF, there was no significant differences in the mean sperm concentration.

### Extracellular vesicles characterization by TEM

Among the animals analyzed, three animals from each group (F and SF) were selected regarding the most representative differences in fertility and semen tests: fertility status ($P < 0.0001$; F: 97.44 ± 0.51%; SF: 30.45 ± 3.38%), means % motile sperm ($P < 0.001$; F: 66.83 ± 2.37%; SF: 14.38 ± 5.60%), mass motility ($P < 0.001$; F: 6.94 ± 0.24; SF: 2.33 ± 0.19) and concentration ($P < 0.01$; F: $3.01 \times 10^9$ sperm/mL ± 0.18; SF: $1.88 \times 10^9$ sperm/mL ± 0.13). From these animals, EVs-enriched samples were observed by transmission electron-microscopy. EVs presented the characteristic morphology of nanometric extracellular rounded and chapped vesicles in F and SF animals. Size characterization revealed EVs of 25–444 nm in F roosters and of 17–348 nm in SF animals (Fig. 1A and B) with a mean diameter of 65.12 nm for F roosters and 77.18 nm for SF animals (Fig. 1B). EVs size differential analysis showed distinct distribution between groups (Fig. 1C). Seminal plasma EVs of diameter less or equal to 70 nm were more abundant in F than in SF roosters (Fig. 1C). By contrast, EVs sized 70–100 nm or above 100 nm were more abundant in SF than in F seminal plasma (Fig. 1C).

### Extracellular vesicle protein markers

The measurement of relative signal intensity indicated EVs with significant intense signal of HSP90A ($P < 0.001$) in F animals in comparison with SF roosters (Fig. 2A and B). There were no significant intensity differences between F and SF for the EVs markers ANXA5, PDCD6IP and VCP.





Downloaded from Bioscientifica.com at 04/09/2023 07:56:17AM
via free access

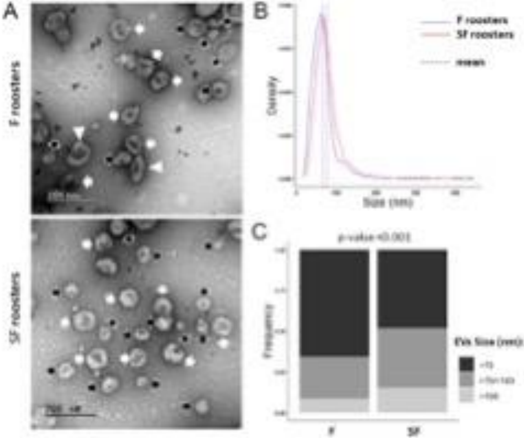


**Figure 1** Characterization by transmission electron-microscopy (TEM) of extracellular vesicles (EVs) isolated from fertile (F) and subfertile (SF) seminal plasma (three animals per condition). (A) EVs sizes 20–70 nm (black arrow), 71 < 100 nm (white arrow), and >100 nm (white arrow-head) were observed in seminal plasma from F and SF roosters. (B) Representative graphic of EVs size distribution in experimental groups (F in blue and SF in red). Dotted lines indicate the mean size of characterized EVs in each experimental group (F and SF). (C) Frequency analysis of the EVs size (<70 nm, 70 < 100 nm, and >100 nm) analyzed by chi² test.

### *Extracellular vesicles incorporation by sperm*

*In vitro* experiments were performed by EVs-sperm co-incubation assays on 3 F and 3 SF animals regarding the most representative differences in fertility and semen tests, as described above. EVs incorporation into sperm cells were observed predominantly in the head of the sperm and a faint signal in the intermediate piece. On the head of the sperm, intense droplets signals could be observed. No signal was observed on the sperm tail (Fig. 3A).

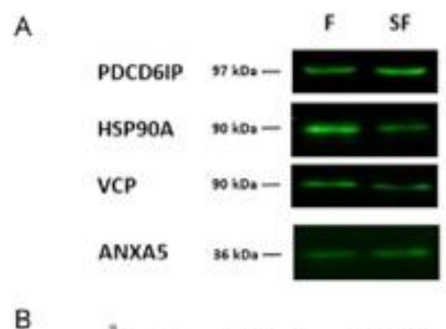
Co-incubation experiments revealed significant impacts of the sperm origin, of the EVs-enriched fraction origin and a significant interaction between these two variables on the PKH67 staining (Fig. 3B, 3 roosters per fertility status, in triplicate). Indeed, the highest PKH67 staining was observed in F sperm co-incubated with EVs isolated from F animals ($P < 0.001$). Furthermore, PKH67 staining was lower in sperm of SF compared to sperm of F rooters, when cultured with EVs from F chickens. No significant impact of the EVs origin on PKH67 staining was revealed in SF sperm, presenting lower staining than F sperm.

In order to better understand the EVs incorporation in F sperm, we performed a new statistical analysis of the experimental data obtained from the previous experiment, focusing on conditions containing F sperm and including a new condition of F sperm in absence of EVs (Fig. 3C). A significant impact of EVs presence was observed, supported by a higher PKH67 staining in experimental conditions with EVs when compared to F sperm alone ($P < 0.001$), confirming that the PKH67 staining was linked to the presence of EVs. As revealed by previous statistical model, the PKH67 staining of F sperm was higher with EVs from F animals than in all other conditions ($P < 0.001$).

### *Sperm viability and motility in presence of extracellular vesicles*

The presence of 10 µg of EVs-enriched fraction had no impact on the viability of F or SF sperm. The dose of 50 µg significantly increased the viability of F sperm after 15 min of co-incubation ($P < 0.001$) (Fig. 4).

On F sperm, there were significant impacts of the experiment duration and of the interaction between the experiment duration and the EVs presence, with higher F sperm viability at 15 min in presence of EVs when compared to without EVs ($P < 0.001$). Concerning the



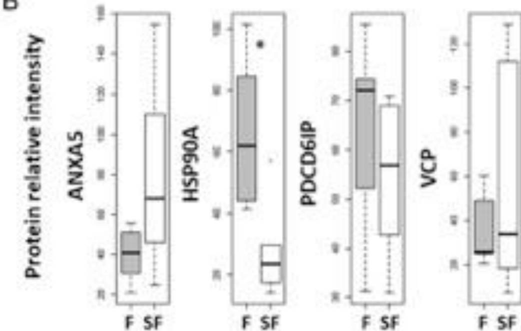




**Figure 2** Western blotting results of ANXA5, HSP90A, PDCD6IP and VCP protein abundances in extracellular vesicles (EVs) isolated of seminal plasma from fertile (F) and subfertile (SF) roosters (six animals per condition, in triplicate). (A) Signal images obtained by Western blotting using specific antibodies. (B) Boxplots of the relative signal intensity of protein EVs markers immunodetected from F (gray bar) and SF (white bar) animals. Protein relative abundance intensity (mean of intensities of Western blotting quantifications performed from six animals per group) as arbitrary units normalized on the total quantity of proteins loaded. Internal black line in the bars indicates the median of the intensities of the experimental group and (*) indicates the statistical difference between the groups ($P < 0.001$).



Downloaded from Bioscientifica.com at 04/08/2023 07:56:17AM
via free access

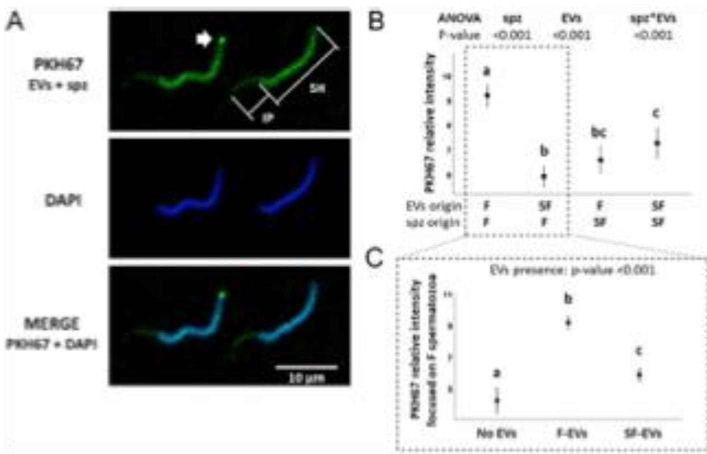



**Figure 3** Incorporation in rooster sperm of extracellular vesicles (EVs) marked with PKH67 lipophilic stain (three roosters per fertility status, in triplicate). (A) Sperm head (SH) and intermediate piece (IP) stained after 1 h co-incubation with 10 µg EVs isolated from fertile (F) animals and PKH67-stained (PKH67, green) on F sperm. Sperm nucleus were stained with DAPI (blue). Merging image were resultant from PKH67 and DAPI signal images (Merge). Arrow indicates dropped concentration of green signal. (B) Representative graphic of EVs PKH67-stained from F and subfertile (SF) animals incorporated by F and SF sperm, after 1 h co-incubation. (C) Detail of incorporation intensities in F sperm incubated with (isolated from F or SF animals) or without EVs (No EVs). PKH67 emission intensities quantification was normalized by DAPI signals. Significant difference in pairwise *post hoc* analysis between conditions were expressed by different letters ($P < 0.05$). Scale bare (10 µm).

SF sperm, only a significant impact of the experiment duration was observed with the 50 µg dose ($P < 0.008$), as revealed by a general increase of the SF sperm viability.

A difference of proportion of motile sperm was observed between F and SF sperm with a higher motility observed in F sperm (Fig. 5). For a given time, the supplementation with 10 µg of EVs-enriched fraction isolated from F animals did not significantly affect the proportion of motile sperm, but the dose of 50 µg induced a significant reduction of the percentage of F motile sperm after 15 min of incubation ($P < 0.001$). This effect was not observed with SF sperm. The duration of the experiment significantly affected the proportion of motile sperm with a decrease for F sperm ($P < 0.001$), and more complicated time-EVs interactions effect in SF sperm.

The 10 µg dose showed a significant effect of interaction between experiment duration and EVs presence in F sperm supported by higher motility in F sperm with EVs at 5 min ($P < 0.001$) when compared to the samples without EVs at the same time, whereas the higher motility was observed in samples without EVs at 15 min.

## Discussion

During the last two decades, seminal EVs have been largely studied in mammals (Sullivan *et al.* 2005), at the difference of other animal classes such as birds. In the present study, we were able to demonstrate, for the first time, the presence of EVs in the rooster seminal fluid. We could also report a difference of seminal EVs size

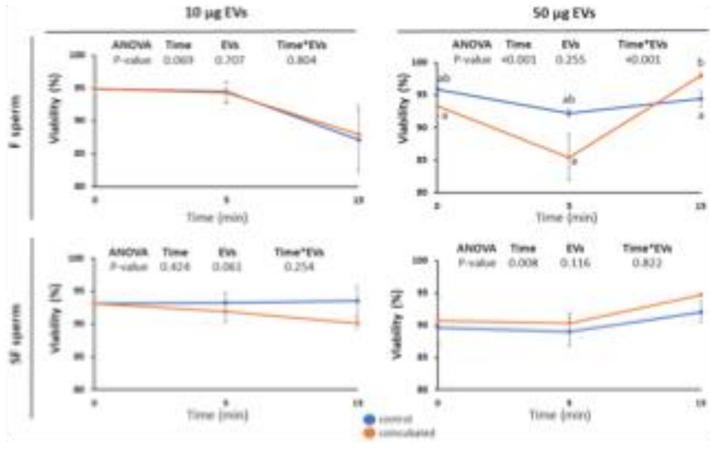



**Figure 4** Sperm viability (three animals per conditions, in triplicate) evaluated by flow cytometry after incubation at 37°C in the presence of 10 or 50 µg of extracellular vesicles (EVs, red lines) isolated from seminal plasma of fertile animals during 0, 5 and 15 min. The control without EVs is represented in blue lines. Data are presented as the average of the percentages (%) and standard deviation. Significant difference in pairwise *post hoc* analysis between conditions were expressed by different letters ($P < 0.05$).



Downloaded from Bioscientifica.com at 04/09/2023 07:56:17AM
via free access

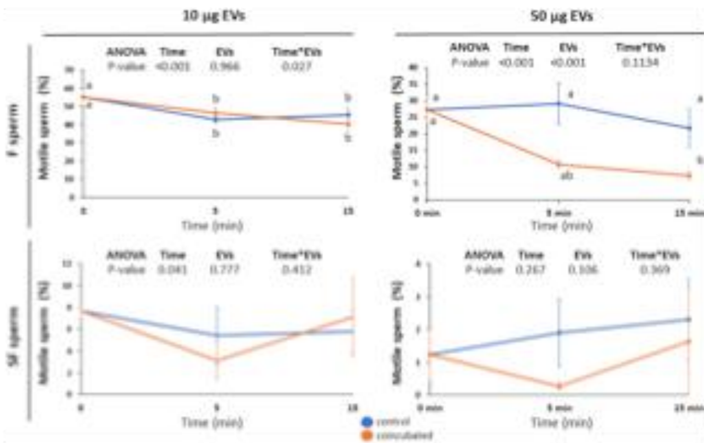


**Figure 5** Sperm motility analysis from fertile (F) and subfertile (SF) roosters (three animals per conditions, in triplicate) co-incubated with 10 µg or 50 µg of extracellular vesicles (EVs, red lines) isolated from seminal plasma of fertile animals during 0, 5 and 15 min. Percentage (%) of motile sperm evaluated by CASA. The control without EVs is represented in blue lines. Data are presented as the average of the percentages (%) and S.D. Significant difference in pairwise *post hoc* analysis between conditions were expressed by different letters ($P < 0.05$).

between the most contrasted F and SF roosters. These EVs expressed classical EVs protein markers and among the tested ones, HSP90A content was significantly higher in F roosters when compared to SF animals. Our incorporation experiments also revealed a difference of EVs fusion to sperm depending on fertility status. This incorporation could be involved in the observed modulation of sperm motility in presence of EVs.

Despite the presence of EVs protein markers in seminal plasma (Labas *et al.* 2015, Borziak *et al.* 2016, Li *et al.* 2020), Alvarez-Rodrigues *et al.* (2020) presented EVs as potentially very rare in this fluid in chickens. It seems clear that the observation of EVs is not easy to reveal in chickens due to specific features of the species (high sperm metabolic rate, rapid degradation after semen collection, high sensitivity of the membranes to physical disruption) (Lemoine *et al.* 2008, Blesbois 2012, Nguyen *et al.* 2014). Thus, in order to succeed in the observation of seminal EVs in roosters, we built a protocol of extraction including immediate use of antiproteases, successive centrifugations and successive accelerations adapted to rooster semen, in order to preserve the EVs structures.

With this approach, we successfully isolated and observed round shaped EVs in rooster seminal plasma of F and SF animals. These EVs are predominantly smaller than 100 nm of diameter, suggesting a large proportion of small EVs (such as exosomes) in the chicken seminal EVs population (Van Niel *et al.* 2018) as observed in mammalian species such as boars (Alvarez-Rodriguez *et al.* 2019, Barranco *et al.* 2019) and humans (Aalberts *et al.* 2012). However, recent evidence suggested that size definition of EVs was not enough to define the different EVs subpopulations (such as exosomes or microvesicles) (Van Niel *et al.* 2018). The EVs classification also relies on the cellular compartment involved in their biogenesis, that is, inner cell part for exosomes and cellular plasma membrane for microvesicles (Van Niel *et al.* 2018). Consequently, further analyses will be necessary to precisely evaluate the different EVs subpopulations, especially by identifying their biogenesis cellular location.

Furthermore, our data revealed a difference of chicken seminal EVs size distribution according to the individual fertility status, with smaller EVs in F animals. No EVs size difference has been observed between seminal plasma EVs from patients with normospermia when compared to individuals with asthenozoospermia (Murdica *et al.* 2019). Our data suggest a modification of the EVs production or subtypes according to the sperm fertility ability.

In order to precise seminal EVs, we investigated the presence of classical EVs markers, including ANXA5, HSP90A, PDCD6IP and VCP (Bobrie *et al.* 2012, Théry *et al.* 2018, Almiñana & Bauersachs 2019, Riou *et al.* 2020). All of them were present in the EVs-enriched fractions. One of these investigated proteins, HSP90A is reported as expressed in the sperm cells of various species (Yue *et al.* 1999) including chickens (Soler *et al.* 2016). HSP90A expression is crucial for the male fertile since its depletion in *Drosophila* is associated to male sterility, relative to a motility reduction (Yue *et al.* 1999). Consequently, we can hypothesize that chicken EVs are involved in the presence or maintenance of HSP90A in the sperm. Moreover, HSP90A was present in higher amount in the EVs of F males when compared to SF animals. Since HSP90A content was previously reported to be higher in the sperm cells of subfertile males (Soler *et al.* 2016), by opposite to seminal EVs, this suggest complex mechanisms of exchanges and integration of EVs information by the sperm.

Even if EVs isolated from F and SF males were efficient to incorporate F sperm, this efficiency was higher concerning EVs-enriched fraction extracted from F roosters. This EVs incorporation was predominantly observed in rooster sperm head as already observed



Downloaded from Bioscientifica.com at 04/09/2023 07:56:17AM
via free access

in mammals (Arienti *et al.* 1997, 2004, Du *et al.* 2016, Zhou *et al.* 2019). This suggests a difference of the EV cell-targeting signal according to the male fertility status. Furthermore, no differential EVs incorporation has been revealed in SF sperm. Consequently, since the EVs isolated from the F sperm could merge with F sperm, the little incorporation of EVs into SF sperm suggests a perturbation of the fusion mechanisms. On one hand, due to the putative role of EVs in exchanges between the sperm and their extracellular environment, this may suggest a reduction of these exchanges in case of SF sperm and thus a perturbation of the sperm maturation process. On the other hand, we can hypothesize that F animals contain a predominant subtype of EVs, with different protein content and greater capacity for incorporation, as suggested for the impact of female physiological status on endometrial EVs incorporation into spermatozoa (Murdica *et al.* 2020), helping them to keep high fertilization capacity.

These hypotheses are consistent with the absence of EVs impact on sperm motility and viability of SF animals, by opposite to F sperm. Thus, the EVs presence could not reestablish a normal percentage of motile sperm in SF animals. However, the absence of EVs impact on SF sperm motility and viability do not conclude of the total absence of EVs impact on SF biology. Other effects need to be further explored such as the impact on acrosome reaction (Murdica *et al.* 2019, 2020).

Interestingly, in addition to the dose-dependent response of F sperm to EVs (observed only with 50 µg EVs-enriched fraction), the sperm viability of F males increased after 15 min of incubation while motility decreased. At 37°C, incubation of bird sperm is still a delicate operation since the rooster sperm are extremely active at this temperature and may lose very fast their fertilizing capacity. In this condition, it seems that the dose of 50 µg EVs isolated from F individuals may be helpful to maintain the sperm viability. The role of EVs in the regulation of sperm function before fertilization has been reviewed by Barkalina *et al.* (2015) and Machtinger *et al.* (2016). Particularly, modification of the sperm motility in the presence of EVs was already reported in humans (Murdica *et al.* 2019), porcines (Alcântara-Neto *et al.* 2020) and boars (Du *et al.* 2016). In chickens, we can hypothesize that the observed decrease of motility could contribute to the maintenance of viability by lowering a too intense metabolic activity of rooster sperm, allowing longer storage or increased survival capacity. However, since, we used successive and differential (ultra)centrifugation approach to reveal seminal EVs, our study of EVs-sperm co-incubation must be confirmed by further different approaches in the future. Indeed, EVs may be isolated by different methods. All of them show specific advantages and limits that may affect their content and functionalities (Doyle & Wang 2019, Jeon *et al.* 2020). Thus, complementary approaches based on other EVs separation technics will be necessary to confirm our functional hypothesis.

In conclusion, we successfully demonstrated the presence of EVs in rooster seminal plasma, with a differential size distribution, protein compositions and efficient incorporation to sperm. These results share new information helpful for further comprehension of the mechanisms involved in sperm fertilizing capacities and for the exploration of new phenotypic tools of reproduction capacity of males.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

## Funding

This study was supported by the European Union's Horizon H2020 Research and innovative Program IMAGE under the grant number 677353 and by the French National Infrastructure of Research CRB Anim funded by 'Investissements d'avenir' (ANR-11-INBS-0003).

## Author contribution statement

L C and E B designed the experiments; L C, I G, H H L L and R U performed the experiments; L C and A V C analyzed the results; L C, A V C and E B wrote the manuscript. All authors approved the final manuscript.

## Acknowledgements

The authors are grateful to the personnel of avian experimental unit (UE-PEAT, INRAE, Nouzilly) in special to Celeste Le Bourhis, and Philippe Didier. The authors would like to express our gratitude to Joël Gautron for kindly providing some of the antibodies and to Guillaume Tsikis for his technical expertise.

## References

**Aalberts M, van Dissel-Emiliani FM, van Adrichem NP, van Wijnen M, Wauben MH, Stout TA & Stoorvogel W** 2012 Identification of distinct populations of prostasomes that differentially express prostate stem cell antigen, annexin A1, and GLIPR2 in humans. *Biology of Reproduction* **86** 82. (https://doi.org/10.1095/biolreprod.111.095760)

**Alcântara-Neto AS, Schmaltz L, Caldas E, Blache MC, Mermillod P & Almiñana C** 2020 Porcine oviductal extracellular vesicles interact with gametes and regulate sperm motility and survival. *Theriogenology* **155** 240–255. (https://doi.org/10.1016/j.theriogenology.2020.05.043)

**Almiñana C & Bauersachs S** 2019 Extracellular vesicles in the oviduct: progress, challenges and implications for the reproductive success. *Bioengineering* **6** 2–25. (https://doi.org/10.3390/bioengineering6020032)

**Alvarez-Rodriguez M, Ljunggren SA, Karlsson H & Rodriguez-Martinez H** 2019 Exosomes in specific fractions of the boar ejaculate contain CD44: a marker for epididymosomes? *Theriogenology* **140** 143–152. (https://doi.org/10.1016/j.theriogenology.2019.08.023)



Downloaded from Bioscientifica.com at 04/09/2023 07:56:17AM via free access

**Alvarez-Rodriguez M, Ntzouni M, Wright D, Khan KI, López-Béjar M, Martinez CA & Rodriguez-Martinez H** 2020 Chicken seminal fluid lacks CD9- and CD44-bearing extracellular vesicles. *Reproduction in Domestic Animals* **55** 293–300. (https://doi.org/10.1111/rda.13617)

**Arienti G, Carlini E & Palmerini CA** 1997 Fusion of human sperm to prostasomes at acidic pH. *Journal of Membrane Biology* **155** 89–94. (https://doi.org/10.1007/s002329900160)

**Arienti G, Carlini E, Saccardi C & Palmerini CA** 2004 Role of human prostasomes in the activation of spermatozoa. *Journal of Cellular and Molecular Medicine* **8** 77–84. (https://doi.org/10.1111/j.1582-4934.2004.tb00261.x)

**Barkalina N, Jones C, Wood MJ & Coward K** 2015 Extracellular vesicle-mediated delivery of molecular compounds into gametes and embryos: learning from nature. *Human Reproduction Update* **21** 627–639. (https://doi.org/10.1093/humupd/dmv027)

**Barranco I, Padilla L, Parrilla I, Álvarez-Barrientos A, Pérez-Patiño C, Peña FJ, Martínez EA, Rodriguez-Martínez H & Roca J** 2019 Extracellular vesicles isolated from porcine seminal plasma exhibit different tetraspanin expression profiles. *Scientific Reports* **9** 11584. (https://doi.org/10.1038/s41598-019-48095-3)

**Blesbois E** 2012 Biological features of the avian male gamete and their application to biotechnology of conservation. *Journal of Poultry Science* **49** 141–149. (https://doi.org/10.2141/jpsa.011120)

**Blesbois E & Brillard JP** 2007 Specific features of in vivo and in vitro sperm storage in birds. *Animal* **1** 1472–1481. (https://doi.org/10.1017/S175173110700081X)

**Blesbois E, Grasseau I, Seigneurin F, Mignon-Grasteau S, Saint Jalme M & Mialon-Richard MM** 2008 Predictors of success of semen cryopreservation in chickens. *Theriogenology* **69** 252–261. (https://doi.org/10.1016/j.theriogenology.2007.09.019)

**Bobrie A, Colombo M, Krumeich S, Raposo G & Théry C** 2012 Diverse subpopulations of vesicles secreted by different intracellular mechanisms are present in exosome preparations obtained by differential ultracentrifugation. *Journal of Extracellular Vesicles* **1** 1–11. (https://doi.org/10.3402/jev.v1i0.18397)

**Borziak K, Álvarez-Fernández A, Karr TL, Pizzari T & Dorus S** 2016 The Seminal fluid proteome of the polyandrous Red junglefowl offers insights into the molecular basis of fertility, reproductive ageing and domestication. *Scientific Reports* **6** 1.

**Brillard JP & McDaniel GR** 1985 The reliability and efficiency of various methods for estimating spermatozoa concentration. *Poultry Science* **64** 155–158. (https://doi.org/10.3382/ps.0640155)

**Burden HP, Holmes CH, Persad R & Whittington K** 2006 Prostasomes – their effects on human male reproduction and fertility. *Human Reproduction Update* **12** 283–292. (https://doi.org/10.1093/humupd/dmi052)

**Burrows WH & Quinn JP** 1937 The Collection of spermatozoa from the domestic fowl and turkey. *Poultry Science* **16** 19–24. (https://doi.org/10.3382/ps.0160019)

**Buschow SI, Van Balkom BWM, Aalberts M, Heck AJR, Wauben M & Stoorvogel W** 2010 MHC class II-associated proteins in B-cell exosomes and potential functional implications for exosome biogenesis. *Immunology and Cell Biology* **88** 851–856. (https://doi.org/10.1038/icb.2010.64)

**Chan JC, Morgan CP, Adrian Leu N, Shetty A, Cisse YM, Nugent BM, Morrison KE, Jašarević E, Huang W, Kanyuch N *et al.*** 2020 Reproductive tract extracellular vesicles are sufficient to transmit intergenerational stress and program neurodevelopment. *Nature Communications* **11** 1499. (https://doi.org/10.1038/s41467-020-15305-w)

**Chen H, Yang P, Chu X, Huang Y, Liu T, Zhang Q, Li Q, Hu L, Waqas Y, Ahmed N *et al.*** 2016 Cellular evidence for nano-scale exosome secretion and interactions with spermatozoa in the epididymis of the Chinese soft-shelled turtle, Pelodiscus sinensis. *Oncotarget* **7** 19242–19250. (https://doi.org/10.18632/oncotarget.8092)

**Conine CC, Sun F, Song L, Rivera-Pérez JA & Rando OJ** 2018 Small RNAs gained during epididymal transit of sperm are essential for embryonic development in mice. *Developmental Cell* **46** 470–480.e3. (https://doi.org/10.1016/j.devcel.2018.06.024)

**Doyle LM & Wang MZ** 2019 Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. *Cells* **8** 727. (https://doi.org/10.3390/cells8070727)

**Druart X & de Graaf S** 2018 Seminal plasma proteomes and sperm fertility. *Animal Reproduction Science* **194** 33–40. (https://doi.org/10.1016/j.anireprosci.2018.04.061)

**Du J, Shen J, Wang Y, Pan C, Pang W, Diao H & Dong W** 2016 Boar seminal plasma exosomes maintain sperm function by infiltrating into the sperm membrane. *Oncotarget* **7** 58832–58847. (https://doi.org/10.18632/oncotarget.11315)

**Fabiani R, Johansson L, Lundkvist O & Ronquist G** 1994 Enhanced recruitment of motile spermatozoa by prostasome inclusion in swim-up medium. *Human Reproduction* **9** 1485–1489. (https://doi.org/10.1093/oxfordjournals.humrep.a138735)

**Frenette G, Lessard C & Sullivan R** 2002 Selected proteins of 'Prostasome-like particles' from epididymal cauda fluid are transferred to epididymal caput spermatozoa in bull. *Biology of Reproduction* **67** 308–313. (https://doi.org/10.1095/biolreprod67.1.308)

**Frenette G, Girouard J, D'Amours O, Allard N, Tessier L & Sullivan R** 2010 Characterization of two distinct populations of epididymosomes collected in the intraluminal compartment of the bovine cauda epididymis. *Biology of Reproduction* **83** 473–480. (https://doi.org/10.1095/biolreprod.109.082438)

**Gabrielsen JS & Lipshultz LI** 2019 Rapid progression in our understanding of extracellular vesicles and male infertility. *Fertility and Sterility* **111** 881–882. (https://doi.org/10.1016/j.fertnstert.2019.02.021)

**Jeon H, Kang SK & Lee MS** 2020 Effects of different separation methods on the physical and functional properties of extracellular vesicles. *PLoS ONE* **15** e0235793. (https://doi.org/10.1371/journal.pone.0235793)

**Labas V, Grasseau I, Cahier K, Gargaros A, Harichaux G, Teixeira-Gomes AP, Alves S, Bourin M, Gérard N & Blesbois E** 2015 Qualitative and quantitative peptidomic and proteomic approaches to phenotyping chicken semen. *Journal of Proteomics* **112** 313–335. (https://doi.org/10.1016/j.jprot.2014.07.024)

**Lake PE & Ravie O** 1981 An attempt to improve the fertility of stored fowl semen with certain additives in a basic diluent. *Reproduction, Nutrition, Developpement* **21** 1077–1084. (https://doi.org/10.1051/rnd:19810806)

**Lemoine M, Grasseau I, Brillard JP & Blesbois E** 2008 A reappraisal of the factors involved in in vitro initiation of the acrosome reaction in chicken spermatozoa. *Reproduction* **136** 391–399. (https://doi.org/10.1530/REP-08-0094)

**Lenth RV** 2016 Least-squares means: the R package lsmeans. *Journal of Statistical Software* **69** 1–33.

**Li Y, Sun Y, Ni A, Shi L, Wang P, Isa AM, Ge P, Jiang L, Fan J, Ma H *et al.*** 2020 Seminal plasma proteome as an indicator of sperm dysfunction and low sperm motility in chickens. *Molecular and Cellular Proteomics* **19** 1035–1046. (https://doi.org/10.1074/mcp.RA120.002017)

**Lin Y, Liang A, He Y, Li Z, Li Z, Wang G & Sun F** 2019 Proteomic analysis of seminal extracellular vesicle proteins involved in asthenozoospermia by iTRAQ. *Molecular Reproduction and Development* **86** 1094–1105. (https://doi.org/10.1002/mrd.23224)

**Machtinger R, Laurent LC & Baccarelli AA** 2016 Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation. *Human Reproduction Update* **22** 182–193. (https://doi.org/10.1093/humupd/dmv055)

**Maxwell WM, de Graaf SP, Ghaoui Rel-H & Evans G** 2007 Seminal plasma effects on sperm handling and female fertility. *Society of Reproduction and Fertility Supplement* **64** 13–38. (https://doi.org/10.5661/rdr-vi-13)

**Munuce MJ, Marini PE & Teijeiro JM** 2019 Expression profile and distribution of annexin A1, A2 and A5 in human semen. *Andrologia* **51** e13224. (https://doi.org/10.1111/and.13224)

**Murdica V, Giacomini E, Alteri A, Bartolacci A, Cermisoni GC, Zarovni N, Papaleo E, Montorsi F, Salonia A, Viganò P *et al.*** 2019 Seminal plasma of men with severe asthenozoospermia contain exosomes that affect spermatozoa motility and capacitation. *Fertility and Sterility* **111** 897–908.e2. (https://doi.org/10.1016/j.fertnstert.2019.01.030)

**Murdica V, Giacomini E, Makieva S, Zarovni N, Candiani M, Salonia A, Vago R & Viganò P** 2020 In vitro cultured human endometrial cells release extracellular vesicles that can be uptaken by spermatozoa. *Scientific Reports* **10** 8856. (https://doi.org/10.1038/s41598-020-65517-9)

**Nguyen TMD, Alves S, Grasseau I, Métayer-Coustard S, Praud C, Froment P & Blesbois E** 2014 Central role of 5'-AMP-activated protein kinase in chicken sperm functions. *Biology of Reproduction* **91** 1–15.

**Piepho HP** 2004 An algorithm for a letter-based representation of all-pairwise comparisons. *Journal of Computational and Graphical Statistics* **13** 456–466. (https://doi.org/10.1198/1061860043515)

**Pinheiro JC & Bates DM** 2013 Mixed-effects models in S and S-PLUS. *Journal of the American Statistical Association* **96** 1135–1136.



Downloaded from Bioscientifica.com at 04/08/2023 07:56:17AM
via free access

R Core Team 2017 *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. (available at: http://www.R-project.org/)

**Raposo G & Stoorvogel W** 2013. Extracellular vesicles: exosomes, microvesicles, and friends. *Journal of Cell Biology* **200** 373–383. (https://doi.org/10.1083/jcb.201211138)

**Renneberg H, Konrad L, Dammshäuser I, Seitz J & Aumüller G** 1997 Immunohistochemistry of prostasomes from human semen. *Prostate* **30** 98–106. (https://doi.org/10.1002/(sici)1097-0045(19970201)30:2<98::aid-pros5>3.0.co;2-g)

**Riou C, Brionne A, Cordeiro L, Harichaux G, Gargaros A, Labas V, Gautron J & Gérard N** 2020 Avian uterine fluid proteome: exosomes and biological processes potentially involved in sperm survival. *Molecular Reproduction and Development* **87** 454–470. (https://doi.org/10.1002/mrd.23333)

**Rodriguez-Martinez H, Kvist U, Ernerudh J, Sanz L & Calvete JJ** 2011 Seminal plasma proteins: what role do they play? *American Journal of Reproductive Immunology* **66** (Supplement 1) 11–22. (https://doi.org/10.1111/j.1600-0897.2011.01033.x)

**Ronquist G** 2012 Prostasomes are mediators of intercellular communication: from basic research to clinical implications. *Journal of Internal Medicine* **271** 400–413. (https://doi.org/10.1111/j.1365-2796.2011.02487.x)

**Ronquist G & Brody I** 1985 The prostasome: its secretion and function in man. *Biochimica et Biophysica Acta* **822** 203–218. (https://doi.org/10.1016/0304-4157(85)90008-5)

**Saadeldin IM, Kim SJ, Choi YB & Lee BC** 2014 Improvement of cloned embryos development by co-culturing with parthenotes: a possible role of exosomes/microvesicles for embryos paracrine communication. *Cellular Reprogramming* **16** 223–234. (https://doi.org/10.1089/cell.2014.0003)

**Saez F, Frenette G & Sullivan R** 2003 Epididymosomes and prostasomes: their roles in posttesticular maturation of the sperm cells. *Journal of Andrology* **24** 149–154. (https://doi.org/10.1002/j.1939-4640.2003.tb02653.x)

**Sahlén GE, Egevad L, Ahlander A, Norlén BJ, Ronquist G & Nilsson BO** 2002 Ultrastructure of the secretion of prostasomes from benign and malignant epithelial cells in the prostate. *Prostate* **53** 192–199. (https://doi.org/10.1002/pros.10126)

**Santiago-Moreno J & Blesbois E** 2020 Functional Aspects of Seminal Plasma in Bird Reproduction. *International Journal of Molecular Sciences* **21** 5664. (https://doi.org/10.3390/ijms21165664)

**Sexton TJ** 1977 A new poultry semen extender. 1. Effects of extension on the fertility of chicken semen. *Poultry Science* **56** 1443–1446. (https://doi.org/10.3382/ps.0561443)

**Soler L, Labas V, Thélie A, Grasseau I, Teixeira-Gomes AP & Blesbois E** 2016 Intact cell MALDI-TOF MS on sperm: a molecular test for male fertility diagnosis. *Molecular and Cellular Proteomics* **15** 1998–2010. (https://doi.org/10.1074/mcp.M116.058289)

**Sullivan R, Saez F, Girouard J & Frenette G** 2005 Role of exosomes in sperm maturation during the transit along the male reproductive tract. *Blood Cells, Molecules and Diseases* **35** 1–10. (https://doi.org/10.1016/j.bcmd.2005.03.005)

**Thélie A, Rehault-Godbert S, Poirier JC, Govoroun M, Fouchécourt S & Blesbois E** 2019 The seminal acrosin-inhibitor ClTI1/SPINK2 is a fertility-associated marker in the chicken. *Molecular Reproduction and Development* **86** 762–775. (https://doi.org/10.1002/mrd.23153)

**Théry C, Amigorena S, Raposo G & Clayton A** 2006 Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Current Protocols in Cell Biology* **30** 3.22.1–3.22.29.

**Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK *et al.*** 2018 Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for extracellular vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles* **7** 1535750. (https://doi.org/10.1080/20013078.2018.1535750)

**Van Niel G, D'Angelo G & Raposo G** 2018 Shedding light on the cell biology of extracellular vesicles. *Nature Reviews: Molecular Cell Biology* **19** 213–228. (https://doi.org/10.1038/nrm.2017.125)

**Vickram AS, Samad HA, Latheef SK, Chakraborty S, Dhama K, Sridharan TB, Sundaram T & Gulothungan G** 2020 Human prostasomes an extracellular vesicle – biomarkers for male infertility and prostrate cancer: the journey from identification to current knowledge. *International Journal of Biological Macromolecules* **146** 946–958. (https://doi.org/10.1016/j.ijbiomac.2019.09.218)

**Yue L, Karr TL, Nathan DF, Swift H, Srinivasans S & Lindquist S** 1999 Genetic analysis of viable Hsp90 alleles reveals a critical role in Drosophila spermatogenesis. *Genetics* **151** 1065–1079.

**Zhou W, Stanger SJ, Anderson AL, Bernstein IR, De Iuliis GN, McCluskey A, McLaughlin EA, Dun MD & Nixon B** 2019 Mechanisms of tethering and cargo transfer during epididymosome-sperm interactions. *BMC Biology* **17** 35. (https://doi.org/10.1186/s12915-019-0653-5)

Received 17 August 2020
First decision 6 October 2020
Revised Manuscript received 24 January 2021
Accepted 25 February 2021



Downloaded from Bioscientifica.com at 04/08/2023 07:58:17AM
via free access

附錄二

# New advances in chicken sperm cryopreservation-the issue of glycerol


Hsiu-Lien Herbie Lin[1,2], Isabelle Grasseau[1], Pascal Mermillod[1], Elisabeth Blesbois[1], Anaïs Vitorino Carvalho[1]

[1] INRAE, CNRS, IFCE, Université de Tours, PRC, 37380, Nouzilly, France; [2] Physiology Division, Livestock Research Institute (LRI), COA, 71246, Tainan, Taiwan



25ème Journée Thésards de l'unité PRC INRAE Val de Loire – 16 et 17 Juin 2021


## INTRODUCTION

Currently, glycerol is the most used cryoprotectant for chicken sperm cryopreservation[1]. 11% glycerol is the standard concentration for freezing chicken sperm but needs to be removed before insemination to avoid the contraceptive effect[2]. The mechanisms underlying this contraceptive effect remains unclear. Here we explore how glycerol impacts on sperm motility at 41°C to mimic the temperature in the female tract. Furthermore, we also try to develop a new strategy based on Percoll™ single layer centrifugation (SLC)[3] to remove glycerol prior to insemination in order to facilitate the application of cryopreserved chicken sperm.

## METHODES

1. Chicken semen was diluted with glycerol-Lake PC diluent to final concentration of 0, 1, 2, 6 and 11% glycerol respectively. Diluted semen was incubated at 41°C and assessed sperm motility with the computer-assisted sperm analysis (CASA, HTM-VOS) at 0, 10, 20 and 30 min.
2. Glycerol removal solution (GRS) was prepared from Percoll™ added with 0.2 M sucrose in different concentration (40, 50, 60 and 70%). 0.5 ml of post-thaw semen was layered on top of 2 ml of GRS and centrifuged at 800 g for 15 min at 4 and 20 °C. Sperm pellets were resuspended to assess glycerol concentration with Glycerol Assay Kit (Sigma-Aldrich) and sperm motility with CASA.
3. Data were presented as mean ± SD and analyzed by two-way ANOVA. Significant difference was identified by different letters ($p<0.05$).

## RESULTAT-1 Impacts of glycerol on sperm motility

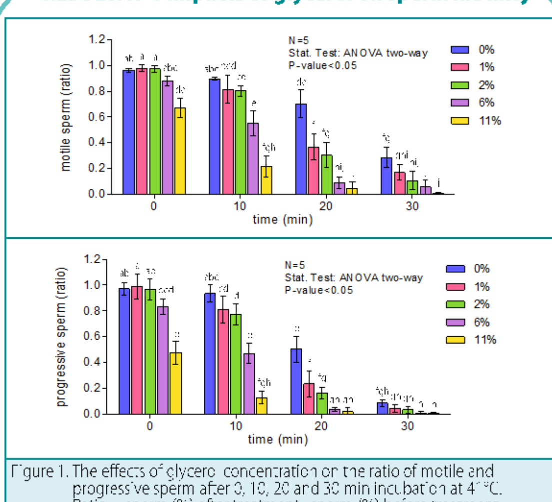



Figure 1. The effects of glycerol concentration on the ratio of motile and progressive sperm after 0, 10, 20 and 30 min incubation at 41°C. Ratio = sperm (%) after treatment : sperm (%) before treatment

1. The ratio of motile and progressive sperm reduced with time, and more reduction appeared when glycerol concentration increased.
2. The ratio of motile sperm of 0, 1 and 2% glycerol sperm was significantly higher than 6 and 11% glycerol sperm at 10 and 20 min, and similar result was obtained for progressive sperm at 10 min.

## RESULTAT-2 Development of new glycerol removal method

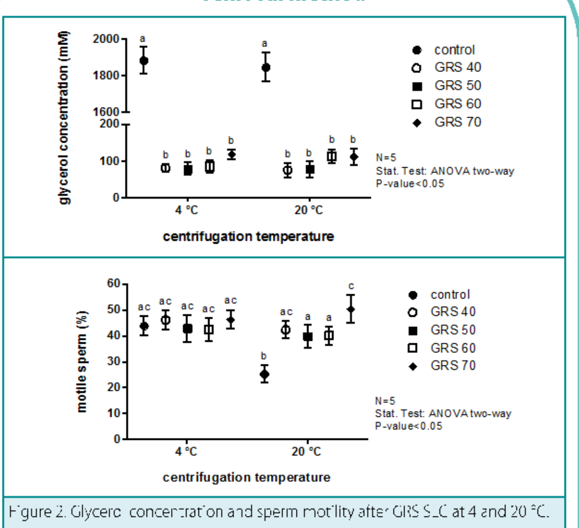



Figure 2. Glycerol concentration and sperm motility after GRS SLC at 4 and 20 °C.

1. GRS SLC efficiently removed glycerol from chicken post-thaw semen.
2. GRS SLC at 20 °C provided a direct selection for motile and progressive sperm (data not shown).

## CONCLUSIONS

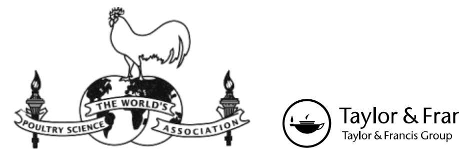
1. Contraceptive effects of glycerol may be partly due to a reduction of sperm motility and thus, a putative impairment of sperm transportation in the female tract. More sperm physiology paramaters (acrosome and mitochondria integrity, apoptosis, ...) are currently investigated to better understand glycerol impact on chicken sperm.
2. Our new glycerol removal method succeed in efficiently removing glycerol saving 44% of time when compared to conventional method (stepwise dilutions followed by centrifugation)[2] and functionally selecting sperm with higher motility which is the parameter strongly correlated with fertility[1]. Further insemination test are needed to ensure the pratical application of this method.

## REFERENCES


1. Abouelezz et al. 2015. Theriogenology 83 (9):1541-1547.
2. Seigneurin and Blesbois. 1995. Theriogenology 43 (8):1351-1358.
3. Lin et al. 2020. British Poultry Science 61 (1):86-91.
4. Blesbois et al. 2008. Theriogenology 69 (2):252-261.

附錄三

WORLD'S POULTRY SCIENCE JOURNAL
2022, VOL. 78, NO. 1, 139–160
https://doi.org/10.1080/00439339.2022.1998816



# Chicken semen cryopreservation: importance of cryoprotectants

Hsiu-Lien Herbie Lin[a,b], Elisabeth Blesbois[a] and Anaïs Vitorino Carvalho[a]

[a]CNRS, IFCE, INRAE, Université de Tours, PRC, Nouzilly, France; [b]Physiology Division, Livestock Research Institute (LRI), COA, Tainan, Taiwan

**SUMMARY**
Sperm cryopreservation is an important strategy to conserve animal genetic diversity and transmit superior genetic background, facilitated by a non-invasive sampling and collection of large amounts of sperm. In chickens, various freezing protocols and cryoprotectants (CPAs) have been developed to freeze sperm, but each lab still debates and competes to disclose the most suitable freezing conditions. Many key points, such as semen donor selection, collection tips, diluent compositions, CPA selection, pre-freezing manipulation, semen packaging type, freezing and thawing rates, are all impactful to the efficiency of chicken sperm cryopreservation. Whereas glycerol is believed as the most efficient and the less toxic CPA, it also presents an unwanted contraceptive effect after insemination. Thus, one of the major developed strategies to conquer this issue is removing glycerol before insemination, but current protocols remain not efficient to completely abolish glycerol contraceptive effect. Novel methodologies will bring us a better understanding of the cellular and molecular mechanisms involved in sperm cryobiology to decipher the challenges of chicken sperm cryopreservation.

**KEYWORDS**
Chicken; semen; freezing; cryoprotectant; glycerol; contraception

## Introduction

Conservation of animal genetic resources is an indispensable strategy to protect biodiversity, to restore endangered breeds or species, to assure animal population from unpredictable epidemics or breeding accidents in animals (Boettcher *et al.* 2013; Mara *et al.* 2013; Joost *et al.* 2015), including fish (Cabrita *et al.* 2010; Torres *et al.* 2016), birds (Blesbois 2011; Rakha *et al.* 2016; Svoradová *et al.* 2018) and mammals (Moore and Hasler 2017; Morrell and Mayer 2017). Conservation of genetic resources for the restoration of genetic diversity can be achieved by different methods of storage of cells able to show a reproductive potential, depending on the species. They mainly include the cryopreservation of sperm, embryos (in mammals), gonadal tissues, and, in specific species, primordial germ cells (chicken)(FAO guideline 2021 *in press)* or stem cells (mouse) (Silva *et al.* 2015). Unlike mammalian species, it is not presently possible to preserve avian oocytes or embryos due to the macrolecithal characteristics of the egg

**CONTACT** Anaïs Vitorino Carvalho Anais.Carvalho@inrae.fr INRAE, CNRS, IFCE, Université de Tours, PRC, Nouzilly 37380, France
© 2021 World's Poultry Science Association.

(Blesbois 2018), including the presence of a very big oocyte (3–4 cm of diameter in the hen) with a very high amount of lipids (yolk) extremely difficult to cryopreserve (Long 2006, 2013; Nakamura 2016). Three germplasm types showing complementary approaches are presently available for cryobanking in poultry: semen, primordial germ cells (PGCs) and gonadal tissue (FAO guideline 2021 *in press)*. Whereas all of these methods show advantages and limits, sperm cryopreservation based on the collection of large amounts of cells in the respect of animal welfare remains the most suitable approach for long-term storage, long-distance genetic exchange and genetic resources management (Çiftci and Aygün 2018; Thélie *et al.* 2019; Cardoso *et al.* 2020). Chicken sperm cryopreservation consists of a long *in vitro* journey for sperm cells including several stages: collection, preparation, freezing and thawing before restoring sperm fertilising capacity and oocyte fertilisation through artificial insemination (AI) process (Figure 1). Whereas current procedures result from more than 60 years of progress, started with the first experiments of semen cryopreservation in 1940s (Shaffner *et al.* 1941) resulting to the first progeny 10 years later using glycerol as CPA (Polge 1951), no standardised protocol has been developed for all chicken breeds/lines. In addition to the relative high cost of sperm freezing and storage compared to maintain the rooster population, and the noticeable decrease in fertility compared to fresh semen, sperm cryopreservation remains in a confidential usage to the progress and commercial application in poultry industry (Donoghue and Wishart 2000). Currently, glycerol is definitely the most broadly used CPA for freezing chicken sperm. It can provide excellent protection for sperm to resist the damages from frozen/thawed processes (Blesbois and Brillard 2007; Blesbois *et al.* 2007; Thélie *et al.* 2019). However, it needs a complex removal procedure before AI. Conversely, CPAs such as dimethyl acetamide (DMA), dimethyl sulphoxide (DMSO) and dimethyl formamide (DMF) are not necessary to be removed prior to AI but give less protection for chicken sperm to oppose the cryodamages.

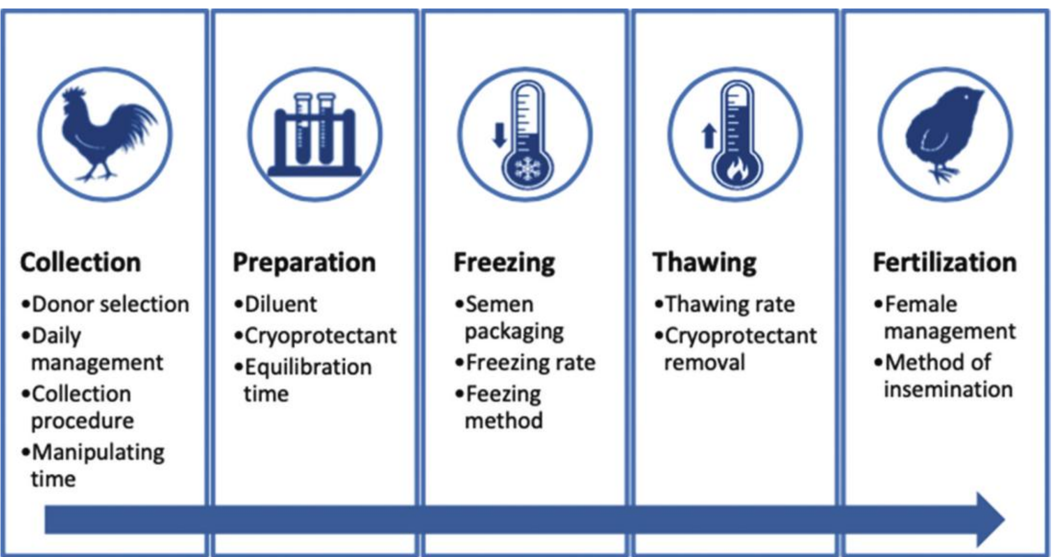



Figure 1. The different stages and influencing factors of chicken sperm cryopreservation.

In this review, we first expose the different main factors impacting chicken sperm cryopreservation, with a special focus on CPAs as one of the major sources of success influence. Subsequently, the issues relative to the historical and widely used CPA, the glycerol, are discussed. Finally, we will present new inspirations for implementing frozen/ thawed chicken semen as a tool for practical reproductive strategy in animal husbandry.

## Critical points of chicken semen cryopreservation

### *Initial semen quality*

#### *Male fertility*

One of the major factors influencing male fertility is the genetic background. Thus, different chicken breeds show different capacities for sperm cryopreservation (Blesbois *et al.* 2007). Another very important point is the male age. Indeed, at the puberty, the young males do not show rapidly an optimal sperm production and quality and then show variations with the annual cycle of reproduction under photoperiodic control (De Reviers *et al.* 1981). This quality increases after some weeks but usually decreases in the second part of the sexual season. As an example, in commercial broiler breeder males reared under daily photoperiod 14 h light:10 h dark and proper management, the optimal age to collect semen for cryopreservation is usually between 30 and 40 weeks (unpublished observations). The length and efficiency of the sexual seasons vary also a lot with external factors including climate, photoperiod, food and water, consequently influencing sperm production and quality (Tabatabaei *et al.* 2010; Shanmugam *et al.* 2014; Rakha *et al.* 2017).

#### *Chicken semen collection*

Chicken semen is generally collected by a non-invasive method, the dorso-abdominal massage (Burrows and Quinn 1937). This method usually involves two persons, one for abdominal massage to make a gentle eversion of the cloacum and to access the opening of the deferent duct and the ejaculation reflex (Mohan *et al.* 2018), and the other for the semen flow into a container. There is a risk of contamination of semen with other cloaca products (i.e. faeces, urates, transparent fluid, reviewed by Etches 1996), which is deleterious to sperm conservation, that could be avoided by the well-training and experienced skills of the collectors (Mohan *et al.* 2018). Another point to reduce the possibility of contamination is temporarily restriction of feed and water (12 hr) before semen collection (Yadav *et al.* 2019), that is why it is recommended to collect in the morning. Furthermore, routinely collecting semen from donors (2–3 times a week) increases semen quality (De Reviers 1973).

#### *Fresh semen quality*

It is necessary to scan fresh semen quality from males to select the candidates for semen collection. *In vitro* quality tests, especially sperm motility (e.g. mass motility and percentage of motile spermatozoa), sperm viability and sometimes membrane fluidity, have been reported to explain 85% of the variation of fertility of cryopreserved sperm in chickens (Blesbois *et al.* 2008).

## *Key points of the cryopreservation process*

After making sure the best animal conditions, the success of chicken sperm cryopreservation depends on several biophysical and biochemical factors (Figure 1).

### *Manipulating time*

Duration of operating chicken semen from collection to freezing highly impacts sperm cryopreservation. Indeed, neat semen stored *in vitro* at an ambient temperature more than 15 min gradually lose the fertility potential (Blesbois and Brillard 2007), due to a combination of factors including very high metabolic, functional and also proteolytic activities (Lemoine *et al.* 2009; Nguyen *et al.* 2014). Thus, the first recommendation is to manage the shorter possible the time between semen collection and freezing (Vasicek *et al.* 2015).

### *Diluents, CPAs and equilibration time*

Semen diluents can supply continuous physicochemical stability to sperm during *in vitro* storage (Blesbois and Brillard 2007). Iso-osmotic (330–450 milliosmoles) and closely natural pH (6.8–7.4) are adjusted by mixing balanced salt/buffer solutions (Blesbois 2012). They usually contain energetic substrates and products expected to make easier the freeze-thaw process (see after). Dilution rates are also important; the rates 1:3 to 1:5 are usually suggested (Sexton 1981; Seigneurin and Blesbois 1995; Santiago-Moreno *et al.* 2011). Dilution rates might lead to different sperm concentrations during freezing. However, few studies discussed this issue in chicken sperm and showed no significant differences in the fertility (Moghbeli *et al.* 2016b; Pérez-Marín *et al.* 2019).

There are two classes of CPAs: the intracellular and the extracellular ones. Intracellular CPAs such as glycerol, dimethyl sulphoxide (DMSO), dimethyl acetamide (DMA), methyl-acetamide (MA), ethylene glycol (EG), dimethyl formamide (DMF) and also some monosaccharides, are amphipathic and low molecular weights components that can diffuse through cell membrane to minimise cell damages by regulating the formation of intracellular ice crystals, and have been used extensively for chicken sperm cryopreservation (Donoghue and Wishart 2000). Extracellular CPAs, e.g. polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), sucrose, trehalose (disaccharides) and raffinose (trisaccharide), generally form a shield surrounding cells, which can protect cells by reducing extracellular ice crystals and making easier the dehydration of the freezing process (Massip *et al.* 2004; Motta *et al.* 2014; Thananurak *et al.* 2019).

Sperm metabolism must be decreased for *in vitro* storage, which is practically achieved by lowering semen temperature (Lake and Ravie 1982; Giesen and Sexton 1983). Notably, semen temperature must be decreased gradually to prevent harmful cold shock effects (Clarke *et al.* 1982; Wishart 1984). A 2–4°C equilibration is usually applied to semen to add the CPAs with the lowest possible interaction with sperm metabolism before freezing and to ensure an equilibration of CPAs and sperm (Blanco *et al.* 2000; Mphaphathi *et al.* 2016). The best equilibration time depends on the CPAs and on the experimental conditions, varying from 1 min with DMA to 1–2 hours with DMSO (Sexton 1981; Santiago-Moreno *et al.* 2011; Zaniboni *et al.* 2014).

### *Semen packaging types*

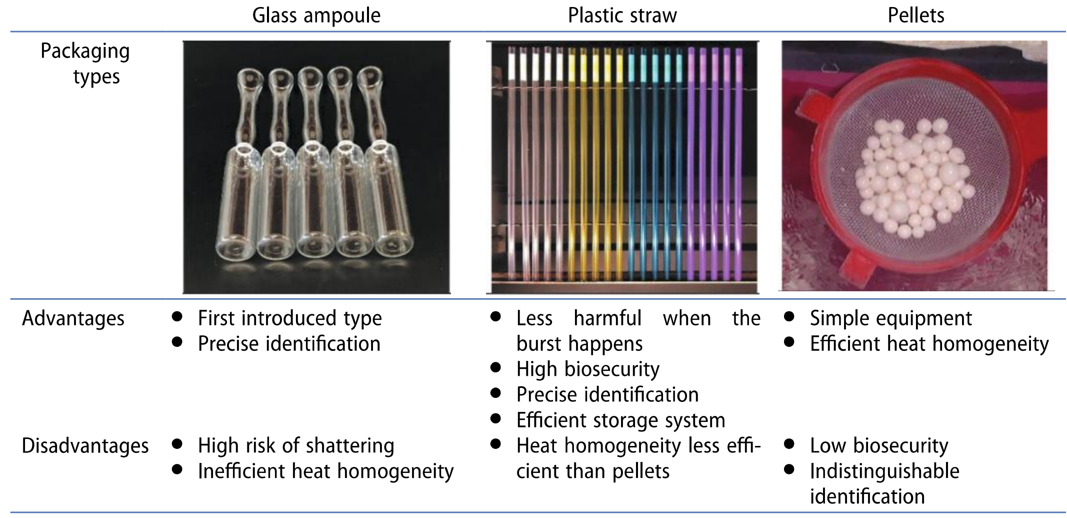
There are three most popular packaging: glass ampoules, plastic straws and pellets (Table 1). Glass ampoule was the first type of container introduced for chicken semen storage (Lake and Stewart 1978). Then, plastic straws and pellets (semen drops) were developed (Sexton 1980; Tselutin *et al.* 1995). Plastic straws facilitate individual identification and storage and also are less harmful than glass ampoule if the burst happens (Pickett and Berndtson 1974; Pickett *et al.* 1978; Mortimer 2004). Pellets are a rapid freezing method, increasing freezing homogeneity compared to plastic straws or glass ampules, and require less equipment (Brotherton 1990). Among these alternatives, considering handling and storing capacity, biosecurity and precise identification of individual animal, plastic straws might be the priority to use for the cryobanking (Tselutin *et al.* 1999; Woelders *et al.* Hiemstra 2006; Blesbois 2007b, 2007a).

Whereas no impact of packaging was observed by Abouelezz *et al.* (2015b), Tselutin *et al.* (1999) reported higher fertility with chicken semen frozen with 6% DMA in pellets than with 6% DMA in straws. The fertility obtained from frozen/thawed semen with 6% DMA in pellets was equivalent to 11% glycerol in straws, which suggested both were optimal combinations of CPAs/packaging. However, the differences between studies might be owing to other side effects (lab conditions, chicken breeds management) confirming that a lot remains to investigate to precisely define the optimal CPA/packaging combination.

### *Freezing rates*

The first really efficient chicken sperm cryopreservation method using glycerol and slow freezing rates, was reported by Lake and Stewart (1978). This method was further improved and one of its adaptation (Seigneurin and Blesbois 1995) was validated for retaining sperm fertilising capacity up to 18 years (Thélie *et al.* 2019). Freezing rates can be conducted by a programmable freezer or by layering semen straws in different distances of the vapour above the surface of liquid nitrogen. Certainly, the former

**Table 1.** Comparisons of different semen packaging types.



| | Glass ampoule | Plastic straw | Pellets |
|---|---|---|---|
| Packaging types | | | |
| Advantages | • First introduced type<br>• Precise identification | • Less harmful when the burst happens<br>• High biosecurity<br>• Precise identification<br>• Efficient storage system | • Simple equipment<br>• Efficient heat homogeneity |
| Disadvantages | • High risk of shattering<br>• Inefficient heat homogeneity | • Heat homogeneity less efficient than pellets | • Low biosecurity<br>• Indistinguishable identification |

method is more precise to control the freezing rates. However, it is expensive and not always available, especially in the field working. Different freezing rates affect post-thaw sperm motility, viability and fertilising ability (Madeddu *et al.* 2016; Thélie *et al.* 2019). Furthermore, optimal freezing rates depends on the CPAs/packaging combination (Sexton 1979). For example, the use of glycerol/straws is conducted with quite low semen freezing rate in the first part of the curve (i.e. 7°C/min up to −35°C), while the use of DMA/pellets may allow a direct plunging in liquid nitrogen. In this last case, the freezing rate depends on the size of the pellet but approximately 600°C/min (Woelders *et al.* 2006). The semen freezing rates suggested for DMF/straws and EG/straws are, respectively, −15 and −1°C/min (Thélie *et al.* 2019).

### Thawing rates

Adjusting thawing rates moderate the water crystals growing and minimise damage to sperm. Chicken semen thawed at 5°C or 60°C, respectively, with semen frozen with glycerol or DMA could lead to desirable fertility (Tselutin *et al.* 1999). Chicken sperm better respond to slow thawing, which depends on freezing rate (Bellagamba *et al.* 1993). Nevertheless, keeping semen at low temperature (5°C) after thawing could be benefit to chicken sperm quality since the infra-physiological temperature might reduce the sperm metabolism (Lake and Ravie 1982; Giesen and Sexton 1983) and the reaction of lipid peroxidation (Fujihara and Howarth JR 1978), and the physiological temperature would be reached only at the time of insemination.

## Critical points related to post thawing conditions

### CPAs removal before AI – glycerol case

The glycerol shows an original effect possibly specific to chicken species, leading an inevitable contraception. One important strategy against glycerol contraceptive effect is to remove it before AI (Table 2). One suggested solution is the dialysis of frozen/thawed fowl semen using Cellophane bags (Polge 1951) or CryoCell® container (Buss 1993). However, this method is time-consuming and causes cell damages due to the imbalanced rates between glycerol and water transport.

Another approach is stepwise dilutions followed by centrifugation (Clark and Shaffener 1960; Lake and Stewart 1978). Although this method is time-consuming (but much less that dialysis) and complex, it has become the most common operation to reduce glycerol concentrations from chicken frozen/thawed semen (Seigneurin and Blesbois 1995; Tselutin *et al.* 1999; Abouelezz *et al.* 2017; Thélie *et al.* 2019) (Figure 2).

**Table 2.** Comparisons of glycerol removal methods.

| Removal methods | Species | Time | Fertility | References |
|---|---|---|---|---|
| Slow dialysis | Chicken | 2 hr | 54% | Polge (1951) |
| CryoCell® dialysis | Chicken | 1.5–2 hr | 56% | Buss (1993) |
| One-step dilutions + centrifugation | Chicken | 7 min | 40% | Clark and Shaffener (1960) |
| Stepwise dilutions + centrifugation | Chicken | 33–45 min | 63% | Lake and Stewart (1978) |
| Stepwise dilutions + centrifugation (simplified) | Chicken | 27 min | 76% | Seigneurin and Blesbois (1995) |
| Accudenz centrifugation | Chicken | 25 min | 37% | Long and Kulkarni (2004) |
| Accudenz centrifugation | Turkey | 25 min | 49% | Long and Kulkarni (2004) |
| Percoll® centrifugation | Turkey | 30 min | 19% | Long and Kulkarni (2004) |

The third method for glycerol removal implied colloidal centrifugation, based on Accudenz or Percoll® solution to separate sperm from glycerolised semen (Long and Kulkarni 2004). Purdy *et al.* (2009) compared the glycerol removal effects of stepwise dilutions and Accudenz centrifugation and showed higher motility obtained with Accudenz procedure but a higher number of recovered sperm with stepwise dilution centrifugation, suggesting efficiency specificities of each procedure. Improvement of concerning colloidal centrifugation is still considered and recently, Lin *et al.* (2020) reported a new protocol for the selection of fresh motile chicken sperm based on Percoll® single-layer centrifugation (SLC). Further investigation will be necessary to confirm if this procedure would be suitable for chicken sperm cryopreserved with glycerol.

### *Methods of AI for frozen/thawed chicken semen*

Intravaginal insemination (IV) (the place of natural mating) is the most common AI technique, developed by Quinn and Burrows (1936). Including welfare-concerned issue, it involves applying gently pressure to the hen's abdomen and everting the vaginal orifice through the cloacum, then sperm deposit into female vagina (3–4 cm deep), allowing normal selection of the best sperm for fertilisation and not disturb the lay. However, to improve the fertility of frozen/thawed chicken semen, other AI methods have been developed, including intrauterine (IU), intramagnal (IM), and intraperitoneal (IP) inseminations (Figure 3). With IU procedure, semen is directly deposed into female uterus by a glass cannula (Allen and Bobr 1955). IM insemination surgically deposits semen into the magnum part of the female oviduct (Bacon *et al.* 1986). With the IP technique, the surgical sperm deposition is performed into peritoneal cavity near the infundibulum (Brown *et al.* 1963). IU, IM and IP show deleterious effects on the laying rate (Allen and Bobr 1955; Brown *et al.* Hobbs 1963; Long and Kulkarni 2004), and IV insemination is also the cheaper technique.

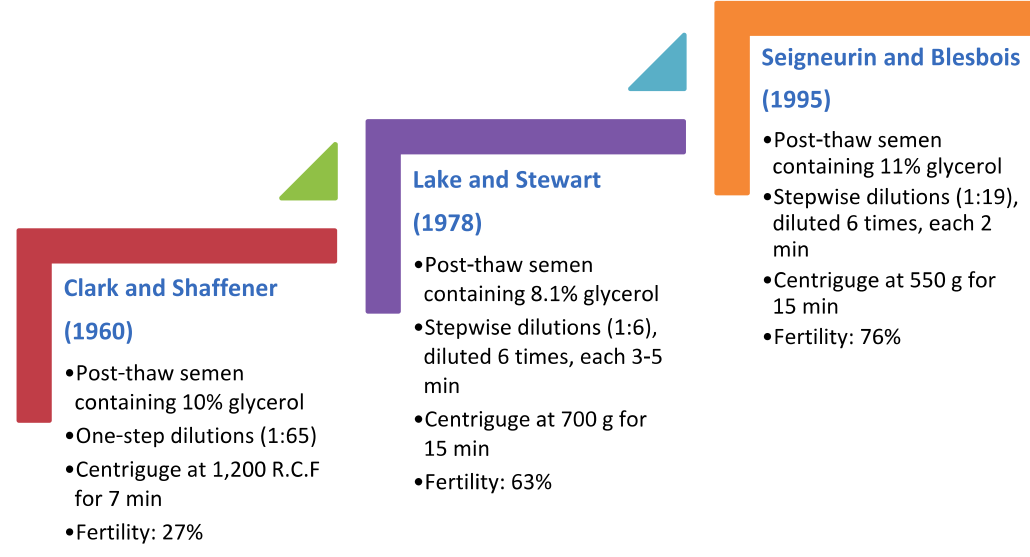



Figure 2. Progress of stepwise dilutions for chicken post-thaw semen.

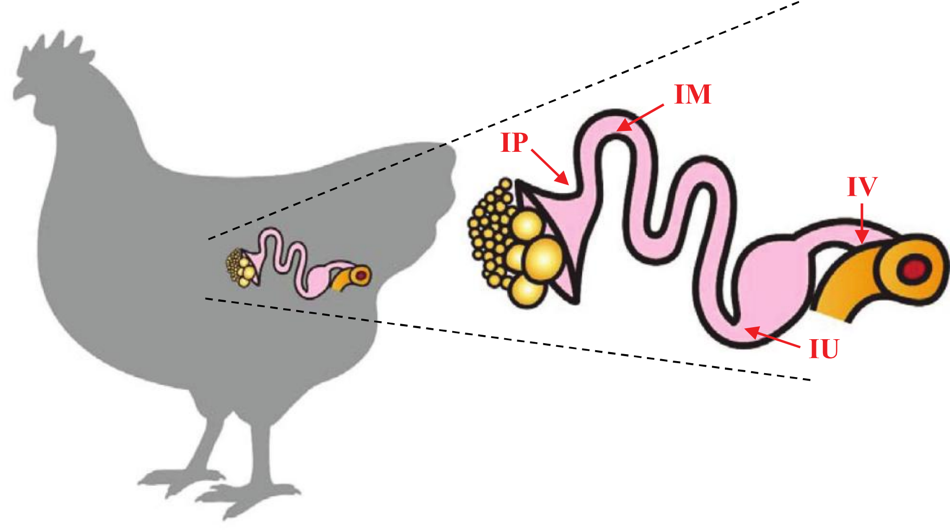



Figure 3. Methods of AI in chicken. Intravaginal (IV), intrauterine (IU), intramagnal (IM) and intraperitoneal (IP) insemination deposit sperm respectively in the vagina, uterus, magnum and peritoneal cavity near the infundibulum.

## The complex choice of cryopreservation medium

As previously described, intracellular CPAs are extensively used for chicken sperm cryopreservation. Their length of entry in sperm is highly variable (one min for glycerol (Lemma 2011) to nearly 3 times longer for DMSO in human sperm (Gilmore *et al.* 1997) and depends also on sperm specificity. Each intracellular CPA can cause specific cytotoxicity (membrane breach, oxidative stress, osmotic shock and chilling injury) (Best 2015), leading no consensus to identify the most suitable CPA for chicken sperm cryopreservation (Table 3).

Whereas Miranda *et al.* (2018) concluded that EG was the most suitable CPA to conserve the motility of frozen/thawed sperm when compared to DMF, DMA and MA; Thélie *et al.* (2019) showed higher fertilising ability of semen frozen/thawed with glycerol when compared to DMF, DMA and EG that showed the lowest results. But Abouelezz *et al.* (2017) showed contrasted effects of glycerol and DMA. The tremendous differences between these results certainly results from complex interactions involving CPAs concentration, extenders, freeze-thaw procedures, animal characteristics and differences in semen quality tests.

In addition to intracellular CPAs, the use of extracellular CPAs, alone or in combination with intracellular CPAs has been explored (Table 3). PVP is classically employed in complement to glycerol (Lake and Stewart 1978; Seigneurin and Blesbois 1995). Egg yolk or its purified components (e.g. egg low-density lipoprotein, LDL) have been used to

Table 3. The effects of various intracellular and extracellular cryoprotectants on chicken frozen/thawed semen.

| *Cryoprotectants* | Diluent | Packaging | Semen quality test | The more recent references |
|---|---|---|---|---|
| *Intracellular only* | | | | |
| 6% DMA | Kobidil | Straw | Motility | Miranda *et al.* (2018) |
| 6% DMF | BHSV | Straw | Fertility | Thélie *et al.* (2019) |
| 7.5% DMF | Kobidil | Straw | Motility | Miranda *et al.* (2018) |
| 8% EG | Kobidil | Straw | Motility | Miranda *et al.* (2018) |
| 10% EG | BHSV | Straw | Fertility | Thélie *et al.* (2019) |
| 9% MA | Kobidil | Straw | Motility | Miranda *et al.* (2018) |
| *Intracellular + extracellular* | | | | |
| 5% DMA + 0.3% PVP | Lake | Pellet | Fertility | Abouelezz *et al.* (2017) |
| 6% DMA + PVP | FEB | Pellet | Fertility | Tselutin *et al.* (1999) |
| 6% DMA + PVP | FEB | Straw | Fertility | Thélie *et al.* (2019) |
| 6% DMA + 0.1 M trehalose + 0.3% PVP | Lake | Straw | Motility | Mosca *et al.* (2016) |
| 6% DMF + 1 mM sucrose | BHSV | Straw | Motility<br>Membrane<br>Acrosome integrity<br>Mitochondria<br>Fertility | Thananurak *et al.* (2019) |
| 2% Glycerol + 4% LDL | Beltsville | Straw | Motility<br>Membrane<br>Mitochondria<br>Fertility | Shahverdi *et al.* (2015) |
| 5% Glycerol + 10% egg-yolk | Ringer lactate | Straw | Motility<br>Viability | Junaedi *et al.* (2016) |
| 8% Glycerol + 0.3% PVP | Lake | Straw | Fertility | Abouelezz *et al.* (2017) |
| 11% Glycerol + 0.3% PVP | Lake | Straw | Fertility | Thélie *et al.* (2019) |
| *Extracellular only* | | | | |
| 15% egg yolk | RFE | Straw | Motility<br>Viability<br>Membrane<br>Acrosome integrity<br>Fertility | Rakha *et al.* (2018) |
| 6% PVP | RFE | Straw | Motility<br>Viability<br>Membrane<br>Acrosome integrity<br>Fertility | Rakha *et al.* (2017) |

DMA: dimethyl acetamide; DMF: dimethyl formamide; EG: ethylene glycol; MA: methyl-acetamide; LDL: low-density lipoprotein; PVP: polyvinylpyrrolidone; RFE: red fowl extender; Kobidil is a commercial diluent; BHSV contains myo-inositol.

cryopreserve successfully domestic chicken semen (Lake 1968; Shahverdi *et al.* 2015; Junaedi *et al.* 2016), or the Red jungle fowl ancestor (Rakha *et al.* 2017, 2018). Moreover, the addition of sucrose to DMF or of trehalose to DMA (Shahverdi *et al.* 2015; Junaedi *et al.* 2016; Mosca *et al.* 2016; Thananurak *et al.* 2019) has been reported to increase post-thaw chicken sperm quality and/or fertility capacities.

### Other potential beneficial additives

Avian sperm membrane contains abundant polyunsaturated fatty acids (PUFAs) (Blesbois *et al.* 1997; Blesbois and Hermier 2003; Cerolini *et al.* 2006; Partyka *et al.* 2012; Fattah *et al.* 2017), which can easily be peroxidised (lipid peroxidation – LPO) in the presence of reactive oxygen species (ROS), leading to membrane, acrosome, mitochondria and DNA disruption (O'Connell *et al.* 2002; Bollwein, Fuchs, and Koess 2008). ROS are active during the frozen/thawed process, increasing the sperm cryoinjuries (Isachenko *et al.* 2003). Antioxidants addition to freezing extenders is a possible solution improving sperm quality after thawing (Salmani *et al.* 2013; Zanganeh *et al.* 2013) (Table 4). Many antioxidants have been tested including vitamins, polyphenols, carotenoids and amino acids (Blesbois *et al.* 1993; Mangiagalli *et al.* 2007; Asmarawati 2010; Tabatabaei *et al.* 2011; Al-Daraji 2014; Moghbeli *et al.* 2016a; Partyka *et al.* 2017; Thananurak *et al.* 2020) with variable results.

Sperm membrane fluidity is a biophysical factor linked to the cholesterol/phospholipid ratio of sperm membrane in addition to membrane PUFAs and others. Freezing is a rigidifying process (Blesbois *et al.* 2005; Chuaychu-Noo *et al.* 2017) and modifying the cholesterol/phospholipid ratio of sperm membrane through the addition of cyclodextrins – cyclic oligosaccharide able to remove or add cholesterol into sperm membranes – may affect positively or negatively semen cryopreservation (Purdy and Graham 2004; Mocé *et al.* 2010). Partyka *et al.* (2016; Partyka *et al.* 2018) used cyclodextrins to increase

**Table 4.** The effects of various beneficial additives on chicken frozen/thawed semen.

| Additive | Diluent | Cryopreotectant | Semen quality test | References |
|---|---|---|---|---|
| *Enhance anti-oxidative capacity* | | | | |
| Vitamin E | Beltsville | 11% glycerol | Motility<br>Viability<br>Membrane | Moghbeli *et al.* (2016a) |
| L-carnitine | EK | 6% DMA | Mitochondria<br>Apoptosis<br>LPO<br>DNA fragmentation | Partyka *et al.* (2017) |
| Hypotaurine | EK | 6% DMA | Mitochondria<br>Apoptosis<br>LPO<br>DNA fragmentation | Partyka *et al.* (2017) |
| Serine | BHSV | 6% DMF | Acrosome integrity<br>Mitochondria<br>Fertility | Thananurak *et al.* (2020) |
| *Adjust membrane fluidity* | | | | |
| 2-hydroxypropyl-β-cyclodextrin | EK | 6% DMA | Motility<br>Mitochondria | Partyka *et al.* (2018) |

the frozen-thawed chicken sperm quality (Table 4). This is opposite to many observations made on mammalian species (Purdy and Graham 2004) and illustrates well the complexity of the parameters involved in membrane fluidity.

### *Specific role of glycerol on chicken sperm cryopreservation*

Historically, 10–20% of glycerol was used to freeze chicken sperm (Polge *et al.* 1949), but the presence of at least 2% glycerol in inseminated semen was associated with contraceptive effect (Polge 1951), leading to the necessary of removing glycerol before AI. Nowadays, 11% glycerol associated to its removal before AI is certainly the most used freezing conditions for chicken sperm cryopreservation (Blesbois and Brillard 2007; Blesbois *et al.* 2007; Abouelezz *et al.* 2015a; Thélie *et al.* 2019). However, despite the development of efficient approach to obtain progeny from chicken sperm frozen with glycerol, the mechanisms underlying the glycerol contraceptive effect are still unclear, but several theories have been proposed, including direct effects on sperm and on female reproductive tract (Figure 4).

#### *Direct impacts of glycerol on sperm biology*

Glycerol (4% and 8%) increased the respiration rate of sperm cells for at least 60 min but reduced sperm motility for only 15 min when chicken sperm were incubated at physiological temperature (41°C) (Sexton 1973), which was not observed with DMSO or EG (Sexton 1974). Glycerol also impacts sperm penetration into the oocyte by reducing the presence of trypsin-like enzyme (TLE) (Johnson *et al.* 1976) involved in sperm penetration of the vitelline membrane of the ovum during fertilisation (Howarth and Digby 1973). However, this reduction of TLE did not correspond to the severe reduction of fertility that may involve other actors of the acrosome reaction pathway (Johnson *et al.* 1976). Indeed, 11% of glycerol in fresh chicken sperm diluents dramatically decreased the ability of sperm to undergo acrosome reaction (Mocé *et al.* 2010). A modification of sperm acrosome reaction capacity induced by glycerol may be one of the causes of the reduction sperm penetration into ovum perivitelline layer. In all experiments, the hatchability/fertility rate was not affected, indicating that glycerol seemingly impaired partial functions for sperm fertilisation but did not disturb embryonic viability (Neville *et al.* 1971; Sexton 1973). These observations do not exclude potential more discreet effects on the development through potential epigenetic changes as we will see in the paragraph IV.

#### *Glycerol impacts on sperm progression in the female reproductive tract*

In general, fowl sperm appear in the uterus within 30 min or in the infundibulum within 1 hr after IV insemination (Bakst *et al.* 1994). However, no sperm was found at the top of oviduct when hens were inseminated by IV with semen containing 15–20% glycerol (Polge 1951), which presumed that glycerol may also impact the sperm evolution into the female reproductive tract, as potentially partly resulting from decrease of sperm motility. However, fertility was partly restored when 15% glycerolised fresh semen was inseminated IU (Allen and Bobr 1955; Allen 1958), indicating the problem was not only due to sperm biology but

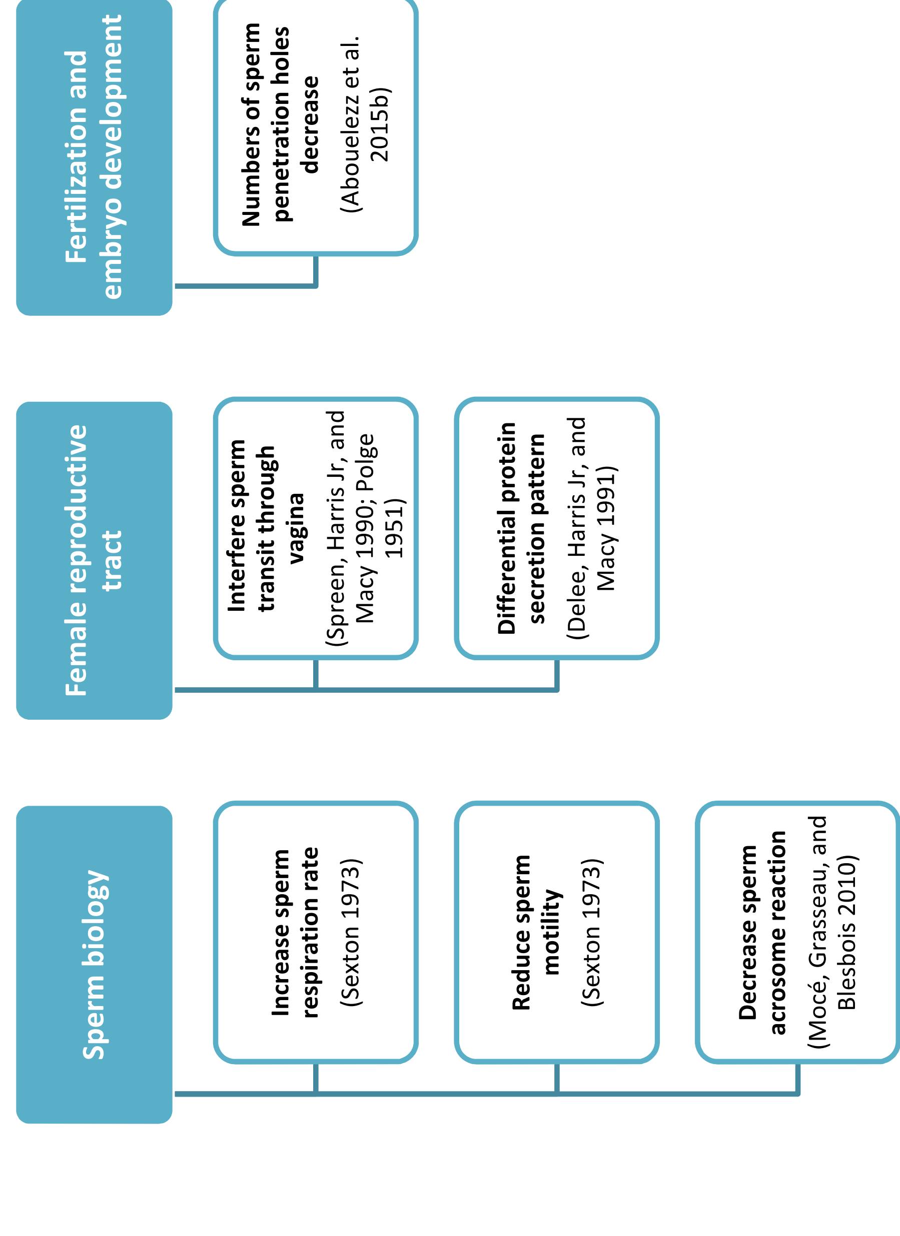



**Figure 4.** Glycerol deleterious effects in chicken reproduction.

also relative to female physiology especially in the lower oviduct part. These authors suggested that the uterovaginal junction might be a barrier to limit glycerolised sperm reaching the upper parts of the oviduct.

Chicken sperm incubated with glycerol and cells derived from female reproductive tract resulted to higher sperm motility and viability, suggesting these cells were beneficial for mitigating cytotoxicity from glycerol (Latorre *et al.* 1988). Spreen *et al.* (1990) indicated the motility of glycerolised sperm significantly decreased in vaginal culture, which was not observed in other reproductive organ-slice cultures, suggesting a specific deleterious effect of glycerol during the sperm transit through vagina. Differential protein secretion pattern was observed in vaginal-slice culture systems in presence or absence of glycerol, but further investigations will be necessary to decipher the proteins associated to the consequence of glycerol on vagina physiology (Delee *et al.* 1991).

Glycerol introduced 5 min before or after fresh semen IV insemination reduced the fertility more in 5 ~ 7 days than in the first 4 days, which suggested that glycerol disturbs sperm transport and/or storage, this is possibly due to an inflammatory response of hen's vagina which would block general sperm transport for a short time (Westfall and Howarth JR 1977). Interestingly, the fertility was not affected when glycerol was inseminated 10 min before semen, which indicated that the antifertility effect of glycerol only would last less than 10 min in the vagina.

### *Is glycerol the only CPA necessary to be removed before insemination?*

Removing glycerol from post-thaw chicken sperm before AI is a necessity procedure. As developed for glycerol, other CPAs may largely influence the sperm transit into female reproductive tract (Harris JR 1968; Mitchell and Buckland 1976), and this point would be questioned for the future. For instance, IU resulted in superior fertility than IV when the DMSO was used (Mitchell and Buckland 1976). IP also led to higher fertility from chicken semen cryopreserved with DMSO than with IV (Harris JR 1968). However, the fertility obtained with these techniques remains lower than with glycerol stepwise dilutions protocol (Brown *et al.* 1963; Seigneurin and Blesbois 1995; Tselutin *et al.* 1999; Thélie *et al.* 2019). Unquestionably, for specific application, such as cryobank programmes when only few offsprings are needed to restore a genetic breed, these techniques may be useful. But bypassing the IV procedure requires well-trained manipulator but also induces much stress to hens which can easily lead to the dramatic drop in egg production and possibility presents other problem such as infections (Allen and Bobr 1955).

## New developments in chicken semen cryopreservation

Improvement in the use of CPAs and their removal after thawing is necessary for the future development of chicken sperm cryopreservation, as well as increasing knowledge in the biochemical mechanisms involved.

We pointed the importance of sperm membrane lipids in the success of freezing, in membrane fluidity demand and biochemical actions (Blesbois *et al.* 1997; Blesbois *et al.* 1997; Douard *et al.* 2000; Blesbois *et al.* 2005), which could possibly be partly re-established by the use of cyclodextrin transporters (Chuaychu-Noo *et al.* 2017; Partyka *et al.* 2018) or other different transporters including lipoprotein complexes.

More discreet mechanisms may be impacted by the cryopreservation procedure. Recent development in omics methods leads to understand better semen molecular actors involved in sperm fertilising ability, such as proteomic studies of chicken semen particularly will be very useful to decipher and identify biomarkers of sperm freezing capacities and fertility (Labas *et al.* 2015). Cheng *et al.* (2015) identified the change in protein abundance in chicken sperm before and after frozen/thawed processes and revealed that most impacted proteins were involved in energy metabolism, hydrolase activity, signal transduction and sperm motility. Other changes have been observed on sperm transcriptome especially on the genes related to proteins involved in stressful conditions such as CIRBP, RHOA, HSP70 and HSP90 (Qi *et al.* 2020). Interestingly, the addition of recombinant HSP90 protein into the extender improved sperm motility and viability, confirming the interest of molecular investigation for the improvement of sperm cryopreservation. Moreover, epigenetic modifications in mature sperm play an important role for the embryo development (Cassuto *et al.* 2016), that may be impacted by sperm cryopreservation as revealed for DNA methylation and histone modifications (Flores *et al.* 2011; Zeng *et al.* 2014; Hezavehei *et al.* 2018). Sperm cryopreservation associated with an elevation of ROS, led to significant reductions of DNA methylation, H3K9 acetylation and H3K4 methylation compared to the fresh semen (Salehi *et al.* 2020). This information could be one clue for the constitution of freezing solutions, including the CPAs, extenders and even antioxidants, which are able to avoid sperm intracellular ROS alterations in epigenetic patterns.

## Conclusion

Sperm cryopreservation is an important strategy to protect animal biodiversity, to transmit superior genetic background and to prevent the animal population devastation from epidemics. It is not yet adapted to commercial chicken industry mainly due to the noticeable reduction of fertilising capacity of chicken sperm after freeze/thaw processes. Glycerol is deemed as the most effective and universal cryoprotectant for chicken sperm, but it can also lead contraception to hens if not removed properly before artificial insemination. In contrast, other frequent cryoprotectants, such as DMA, DMSO, DMF, do not require to be removed before insemination, but are less efficient to protect chicken sperm from cryodamages. Therefore, looking for a novel and powerful sperm cryopreservation procedures based on efficient cryoprotectants adapted to most chicken breeds and able to remove any contraceptive effects is mandatory to make chicken sperm cryopreservation more acceptable in practical industry.

In the past half century, scientists made a lot of efforts to verify the best cryopreservation protocol for chicken sperm. Presently, freezing and storing chicken sperm over a long period of time is no longer a fairy tale. Understanding the cellular and molecular regulations involved in the cryopreservation process can be very useful to optimise the use of frozen/thawed semen. Modern omics technology are very powerful techniques that can dig out abundant and insightful information about the mechanisms related to cryobiology, which may help us to find out the solutions to overcome the obstacles.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

## Funding

This work was supported by the French National Infrastructure of Research CRB anim funded by 'Investissements d'avenir', Agence Nationale de la Recherche [ANR-11- INBS-0003] and from the French National Institute of Agronomic and Environmental Research (INRAe).

## Notes on contributors

*Hsiu-Lien Herbie Lin* works for the projects on sperm quality evaluation and cryopreservation of chickens and pigs at Taiwan Livestock Research Institute since 2007. Now she is doing her PhD study in INRAE, France since September 2019. Her study subjects mainly focus on improving chicken sperm cryopreservation and exploring the impacts of cryoprotectants on chicken sperm physiology.

*Dr. Elisabeth Blesbois* is a French scientist that developed researches in Reproductive Biology and Cryobanking. Her main publications are in the field of animal reproductive cells biology and their applications to the improvements of management of genetic diversity and breeding practice. She coordinated a number of national and international programs in this field. She has led several research teams, was a member of the Board of Directors of the French Institute of Agronomic Research and of the High Council of Biotechnology.

*Anaïs Vitorino Carvalho* After investigating the regulation of various endometrial factors during implantation and the impact of embryo microenvironment on the early development in cattle, Anaïs Vitorino Carvalho started to explore bird physiology, in 2017, by investigating the epigenetic mechanisms involved in the long-term impact and transgenerational inheritance of embryonic environment perturbations in quails. Since 2019, she has focused her work on the better understanding of the mechanisms of chicken sperm fertilizing capacity.

## ORCID

Hsiu-Lien Herbie Lin http://orcid.org/0000-0003-1438-8950

## References

Abouelezz, F., C. Castaño, A. Toledano-Díaz, M. Esteso, A. López-Sebastián, J. Campo, and J. Santiago-Moreno. 2015a. "Sperm–egg Penetration Assay Assessment of the Contraceptive Effects of Glycerol and Egg Yolk in Rooster Sperm Diluents." *Theriogenology* 83 (9): 1541–1547. doi:10.1016/j.theriogenology.2015.02.002.

Abouelezz, F., C. Castaño, A. Toledano–Díaz, M. Esteso, A. López–Sebastián, J. Campo, and J. Santiago–Moreno. 2015b. "Effect of the Interaction between Cryoprotectant Concentration and Cryopreservation Method on Frozen/thawed Chicken Sperm Variables." *Reproduction in Domestic Animals* 50 (1): 135–141. doi:10.1111/rda.12464.

Abouelezz, F., M. Sayed, and J. Santiago-Moreno. 2017. "Fertility Disturbances of Dimethylacetamide and Glycerol in Rooster Sperm Diluents: Discrimination among Effects Produced Pre and Post Freezing-thawing Process." *Animal Reproduction Science* 184: 228–234. doi:10.1016/j.anireprosci.2017.07.021.

Al-Daraji, H. 2014. “Impact of Extender Supplementation with Tomato Juice on Semen Quality of Chicken Semen during Liquid Storage.” *International Journal of Biological Sciences and Applications* 1 (1): 19–23.
Allen, T. 1958. “The Storage of Fowl Semen at Low Temperatures.” In *Proceedings of Australian Society of Animal Production; 2nd Biennial Meeting*, Melbourne, Australia, pp. 118–119.
Allen, T., and L. W. Bobr. 1955. “The Fertility of Fowl Spermatozoa in Glycerol Diluents after Intrauterine Insemination.” *Poultry Science* 34 (5): 1167–1169. doi:10.3382/ps.0341167.
Asmarawati, W. 2010. “The Effect of Adding Vitamin C and E in Native Chicken Semen Extender Storaged at Temperature 4°C on Semen Quality and Egg Fertility.” *Proceedings of International Seminar on Tropical Animal Production (ISTAP)*, Yogyakarta, Indonesia, pp. 308–313.
Bacon, L. D., D. W. Salter, J. V. Motta, L. B. Crittenden, and F. X. Ogasawara. 1986. “Cryopreservation of Chicken Semen of Inbred or Specialized Strains.” *Poultry Science* 65 (10): 1965–1971. doi:10.3382/ps.0651965.
Bakst, M. R., G. Wishart, and J. P. Brillard. 1994. “Oviducal Sperm Selection, Transport, and Storage in Poultry.” *Poultry Science Reviews* 5: 117–143.
Bellagamba, F., S. Cerolini, and L. Cavalchini. 1993. “Cryopreservation for Poultry Semen.” *World’s Poultry Science Journal* 49: 158–166. doi:10.1079/WPS19930013.
Best, B. P. 2015. “Cryoprotectant Toxicity: Facts, Issues, and Questions.” *Rejuvenation Research* 18 (5): 422–436. doi:10.1089/rej.2014.1656.
Blanco, J. M., G. Gee, D. E. Wildt, and A. M. Donoghue. 2000. “Species Variation in Osmotic, Cryoprotectant, and Cooling Rate Tolerance in Poultry, Eagle, and Peregrine Falcon Spermatozoa.” *Biology of Reproduction* 63 (4): 1164–1171. doi:10.1095/biolreprod63.4.1164.
Blesbois, E. 2007a. “Advances in Avian Semen Cryopreservation.” *INRA, France*: 1-7.
Blesbois, E. 2007b. “Current Status in Avian Semen Cryopreservation.” *World’s Poultry Science Journal* 63 (2): 213–222. doi:10.1017/S0043933907001419.
Blesbois, E. 2011. “Freezing Avian Semen.” *Avian Biology Research* 4 (2): 52–58. doi:10.3184/175815511X13069413108523.
Blesbois, E. 2012. “Biological Features of the Avian Male Gamete and Their Application to Biotechnology of Conservation.” *The Journal of Poultry Science* 49 (3): 141–149. doi:10.2141/jpsa.011120.
Blesbois, E. 2018. “Bird Reproduction Overview.” In *Reference Lodule in Life Sciences*, edited by Michael Skinner, 579–585. Amsterdam: Elsevier Science BV.
Blesbois, E., and D. Hermier. 2003. “Specificity of Fatty Acids in Domestic Birds Spermatozoa.” In *Male Fertility and Lipid Metabolism*, eds. Stephaie R. De Vriese and Armond B. Christophe,73–85. Champaign: American Oil Chemist’s Society Press.
Blesbois, E., F. Seigneurin, I. Grasseau, C. Limouzin, J. Besnard, D. Gourichon, G. Coquerelle, P. Rault, and M. Tixier-Boichard. 2007. “Semen Cryopreservation for Ex Situ Management of Genetic Diversity in Chicken: Creation of the French Avian Cryobank.” *Poultry Science* 86 (3): 555–564. doi:10.1093/ps/86.3.555.
Blesbois, E., I. Grasseau, and F. Seigneurin. 2005. “Membrane Fluidity and the Ability of Domestic Bird Spermatozoa to Survive Cryopreservation.” *Reproduction* 129 (3): 371–378. doi:10.1530/rep.1.00454.
Blesbois, E., I. Grasseau, F. Seigneurin, S. Mignon-Grasteau, M. Saint Jalme, and M. M. Mialon-Richard. 2008. “Predictors of Success of Semen Cryopreservation in Chickens.” *Theriogenology* 69 (2): 252–261. doi:10.1016/j.theriogenology.2007.09.019.
Blesbois, E., I. Grasseau, and J. Blum. 1993. “Effects of Vitamin E on Fowl Semen Storage at 4 C.” *Theriogenology* 39 (3): 771–779. doi:10.1016/0093-691X(93)90260-C.
Blesbois, E., and J. P. Brillard. 2007. “Specific Features of in Vivo and in Vitro Sperm Storage in Birds.” *Animal* 1 (10): 1472–1481. doi:10.1017/S175173110700081X.
Blesbois, E., M. Lessire, and D. Hermier. 1997. “Effect of Cryopreservation and Diet on Lipids of Fowl Sperm and Fertility.” *Poultry and Avian Biology Reviews* 8 (3–4): 149–154.
Blesbois, E., M. Lessire, I. Grasseau, J. Hallouis, and D. Hermier. 1997. “Effect of Dietary Fat on the Fatty Acid Composition and Fertilizing Ability of Fowl Semen.” *Biology of Reproduction* 56 (5): 1216–1220. doi:10.1095/biolreprod56.5.1216.

Boettcher, P., J. Martin, G. Gandini, B. Joshi, and J. Oldenbroek. 2013. "In Vivo Conservation of Animal Genetic Resources." In *FAO Animal Production and Health Guidelines*, 1–29. Rome: FAO. https://www.fao.org/3/i3327e/i3327e.pdf

Bollwein, H., I. Fuchs, and C. Koess. 2008. "Interrelationship between Plasma Membrane Integrity, Mitochondrial Membrane Potential and DNA Fragmentation in Cryopreserved Bovine Spermatozoa." *Reproduction in Domestic Animals* 43 (2): 189–195. doi:10.1111/j.1439-0531.2007.00876.x.

Brotherton, J. 1990. "Cryopreservation of Human Semen." *Archives of Andrology* 25 (2): 181–195. doi:10.3109/01485019008987609.

Brown, J., G. Harris JR, and T. Hobbs. 1963. "Effect of Intraperitoneal Insemination on Egg Production and Fertilizing Capacity of Fresh and Frozen Chicken Sperm." *Poultry Science* 42 (4): 810–815. doi:10.3382/ps.0420810.

Burrows, W., and J. Quinn. 1937. "The Collection of Spermatozoa from the Domestic Fowl and Turkey." *Poultry Science* 16 (1): 19–24. doi:10.3382/ps.0160019.

Buss, E. 1993. "Cryopreservation of Rooster Sperm." *Poultry Science* 72 (5): 944–954. doi:10.3382/ps.0720944.

Cabrita, E., C. Sarasquete, S. Martínez–Páramo, V. Robles, J. Beirão, S. Pérez–Cerezales, and M. Herráez. 2010. "Cryopreservation of Fish Sperm: Applications and Perspectives." *Journal of Applied Ichthyology* 26 (5): 623–635. doi:10.1111/j.1439-0426.2010.01556.x.

Cardoso, B., I. Sánchez-Ajofrín, C. Castaño, O. García-Álvarez, M. C. Esteso, A. Maroto-Morales, M. Iniesta-Cuerda, J. J. Garde, J. Santiago-Moreno, and A. J. Soler. 2020. "Optimization of Sperm Cryopreservation Protocol for Peregrine Falcon (Falco Peregrinus)." *Animals* 10 (4): 691. doi:10.3390/ani10040691.

Cassuto, N. G., D. Montjean, J.-P. Siffroi, D. Bouret, F. Marzouk, H. Copin, and M. Benkhalifa. 2016. "Different Levels of DNA Methylation Detected in Human Sperms after Morphological Selection Using High Magnification Microscopy." *BioMed Research International* (2016) (5): 1–7. doi:10.1155/2016/6372171.

Cerolini, S., L. Zaniboni, A. Maldjian, and T. Gliozzi. 2006. "Effect of Docosahexaenoic Acid and α-tocopherol Enrichment in Chicken Sperm on Semen Quality, Sperm Lipid Composition and Susceptibility to Peroxidation." *Theriogenology* 66 (4): 877–886. doi:10.1016/j.theriogenology.2006.02.022.

Cheng, C.-Y., P.-R. Chen, C.-J. Chen, S.-H. Wang, C.-F. Chen, Y.-P. Lee, and S.-Y. Huang. 2015. "Differential Protein Expression in Chicken Spermatozoa before and after Freezing–thawing Treatment." *Animal Reproduction Science* 152: 99–107. doi:10.1016/j.anireprosci.2014.11.011.

Chuaychu-Noo, N., P. Thananurak, V. Chankitisakul, and T. Vongpralub. 2017. "Supplementing Rooster Sperm with Cholesterol-Loaded-Cyclodextrin Improves Fertility after Cryopreservation." *Cryobiology* 74: 8–12. doi:10.1016/j.cryobiol.2016.12.012.

Çiftci, H., and A. Aygün. 2018. "Poultry Semen Cryopreservation Technologies." *World's Poultry Science Journal* 74 (4): 699–710. doi:10.1017/S0043933918000673.

Clark, C. E., and C. Shaffener. 1960. "The Fertilizing Capacity of Frozen Chicken Sperm and the Influence of Related in Vitro Processes." *Poultry Science* 39 (5): 1213–1220. doi:10.3382/ps.0391213.

Clarke, R., T. Sexton, and M. Ottinger. 1982. "Effects of Holding Temperature and Storage Time on Respiratory Rate, Motility, and Fertility of Chicken and Turkey Semen." *Poultry Science* 61 (9): 1912–1917. doi:10.3382/ps.0611912.

De Reviers, M. (1973) "Sperm Transport and Survival in Male Birds." *Proceedings of INSERM International Symposium*, Nouzilly, France, pp. 10–16.

De Reviers, M., J. Williams, M. Garreau, and J. P. Brillard. 1981. "Predicting the Adult Daily Sperm Output after the First Ejaculates in Cockerels Raised under Different Photoschedules." *Reproduction, Nutrition, Development* 21 (6B): 1113–1124. doi:10.1051/rnd:19810810.

Delee, J., G. Harris JR, and L. Macy. 1991. "Research Note: The in Vitro Responses of Vaginal Tissue and Chicken Spermatozoa to Glycerol." *Poultry Science* 70 (6): 1441–1443. doi:10.3382/ps.0701441.

Donoghue, A. M., and G. Wishart. 2000. "Storage of Poultry Semen." *Animal Reproduction Science* 62 (1–3): 213–232. doi:10.1016/S0378-4320(00)00160-3.
Douard, V., D. Hermier, and E. Blesbois. 2000. "Changes in Turkey Semen Lipids during Liquid in Vitro Storage." *Biology of Reproduction* 63 (5): 1450–1456. doi:10.1095/biolreprod63.5.1450.
Etches, R. J. 1996. "Artificial Insemination." In *Reproduction in Poultry*, ed. Robert J. Etches, 234–262. Wallingford: CAB international.
Fattah, A., M. Sharafi, R. Masoudi, A. Shahverdi, V. Esmaeili, and A. Najafi. 2017. "l-Carnitine in Rooster Semen Cryopreservation: Flow Cytometric, Biochemical and Motion Findings for Frozen-thawed Sperm." *Cryobiology* 74: 148–153. doi:10.1016/j.cryobiol.2016.10.009.
Flores, E., L. Ramió-Lluch, D. Bucci, J. Fernández-Novell, A. Peña, and J. Rodríguez-Gil. 2011. "Freezing-thawing Induces Alterations in Histone H1-DNA Binding and the Breaking of protein-DNA Disulfide Bonds in Boar Sperm." *Theriogenology* 76 (8): 1450–1464. doi:10.1016/j.theriogenology.2011.05.039.
Fujihara, N., and B. Howarth JR. 1978. "Lipid Peroxidation in Fowl Spermatozoa." *Poultry Science* 57 (6): 1766–1768. doi:10.3382/ps.0571766.
Giesen, A., and T. Sexton. 1983. "Beltsville Poultry Semen Extender. 9. Effect of Storage Temperature on Turkey Semen Held Eighteen Hours." *Poultry Science* 62 (7): 1305–1311. doi:10.3382/ps.0621305.
Gilmore, J., J. Liu, D. Gao, and J. Critser. 1997. "Determination of Optimal Cryoprotectants and Procedures for Their Addition and Removal from Human Spermatozoa." *Human Reproduction* 12 (1): 112–118. doi:10.1093/humrep/12.1.112.
Harris JR, G. 1968. "Fertility of Chickens Inseminated Intraperitoneally with Semen Preserved in Liquid Nitrogen." *Poultry Science* 47 (2): 384–388. doi:10.3382/ps.0470384.
Hezavehei, M., M. Sharafi, H. M. Kouchesfahani, R. Henkel, A. Agarwal, V. Esmaeili, and A. Shahverdi. 2018. "Sperm Cryopreservation: A Review on Current Molecular Cryobiology and Advanced Approaches." *Reproductive Biomedicine Online* 37 (3): 327–339. doi:10.1016/j.rbmo.2018.05.012.
Howarth, B., and S. Digby. 1973. "Evidence for the Penetration of the Vitelline Membrane of the Hen's Ovum by a Trypsin-like Acrosomal Enzyme." *Reproduction* 33 (1): 123–125. doi:10.1530/jrf.0.0330123.
Isachenko, E., V. Isachenko, I. I. Katkov, S. Dessole, and F. Nawroth. 2003. "Vitrification of Mammalian Spermatozoa in the Absence of Cryoprotectants: From past Practical Difficulties to Present Success." *Reproductive Biomedicine Online* 6 (2): 191–200. doi:10.1016/S1472-6483(10)61710-5.
Johnson, L., T. Sexton, and V. Pursel. 1976. "Effect of Glycerol on Spermatozoa Trypsin-like Enzyme Activity and Fertility in the Chicken." *Theriogenology* 5 (1): 21–27. doi:10.1016/0093-691X(76)90154-0.
Joost, S., M. W. Bruford, and G.-R. Consortium. 2015. "Advances in Farm Animal Genomic Resources." *Frontiers in Genetics* 6. doi:10.3389/fgene.2015.00333.
Junaedi, J., R. I. Arifiantini, C. Sumantri, and A. Gunawan. 2016. "Use of Glycerol as Cryoprotectants in Freezing Sentul Chicken Semen." *Chalaza Journal of Animal Husbandry* 1 (2): 6–13. doi:10.31327/chalaza.v1i2.165.
Labas, V., I. Grasseau, K. Cahier, A. Gargaros, G. Harichaux, A.-P. Teixeira-Gomes, S. Alves, M. Bourin, N. Gérard, and E. Blesbois. 2015. "Qualitative and Quantitative Peptidomic and Proteomic Approaches to Phenotyping Chicken Semen." *Journal of Proteomics* 112: 313–335. doi:10.1016/j.jprot.2014.07.024.
Lake, P. 1968. "Observations on Freezing Fowl Spermatozoa in Liquid Nitrogen." *Proceedings of 6th International Congress of Animal Reproduction and Artificial Insemination*, Paris, France, pp. 1633–1635.
Lake, P., and J. Stewart. 1978. "Preservation of Fowl Semen in Liquid Nitrogen—an Improved Method." *British Poultry Science* 19 (2): 187–194. doi:10.1080/00071667808416462.
Lake, P., and O. Ravie. 1982. "Effect on Fertility of Storing Turkey Semen for 24 Hours at 10 C in Fluids of Different pH." *British Poultry Science* 23 (1): 41–47. doi:10.1080/00071688208447927.

Latorre, J., G. Harris JR, J. Skeeles, and Z. Johnson. 1988. "Effects of Glycerol on Chicken Spermatozoa Incubated in Vitro at 41 C in Oviducal and Embryonic Cell Cultures." *Poultry Science* 67 (1): 135–140. doi:10.3382/ps.0670135.

Lemma, A. 2011. "Effect of Cryopreservation on Sperm Quality and Fertility." *Artificial Insemination in Farm Animals* 12: 191–216.

Lemoine, M., J. Dupont, V. Guillory, S. Tesseraud, and E. Blesbois. 2009. "Potential Involvement of Several Signaling Pathways in Initiation of the Chicken Acrosome Reaction." *Biology of Reproduction* 81 (4): 657–665. doi:10.1095/biolreprod.108.072660.

Lin, H., Y. Chen, D. Lin, Y. Lai, M. Wu, and L. Chen. 2020. "Silica-based Colloid Centrifugation Enhances Sperm Quality in Cockerel Semen." *British Poultry Science* 61 (1): 86–91. doi:10.1080/00071668.2019.1671959.

Long, J. 2006. "Avian Semen Cryopreservation: What are the Biological Challenges?" *Poultry Science* 85 (2): 232–236. doi:10.1093/ps/85.2.232.

Long, J., and G. Kulkarni. 2004. "An Effective Method for Improving the Fertility of Glycerol-exposed Poultry Semen." *Poultry Science* 83 (9): 1594–1601. doi:10.1093/ps/83.9.1594.

Long, J. A. 2013. "028 Successful Cryopreservation of Avian Germplasm: Why a Multifaceted Approach Is Required." *Cryobiology* 67 (3): 406. doi:10.1016/j.cryobiol.2013.09.034.

Madeddu, M., F. Mosca, A. A. Sayed, L. Zaniboni, M. Mangiagalli, E. Colombo, and S. Cerolini. 2016. "Effect of Cooling Rate on the Survival of Cryopreserved Rooster Sperm: Comparison of Different Distances in the Vapor above the Surface of the Liquid Nitrogen." *Animal Reproduction Science* 171: 58–64. doi:10.1016/j.anireprosci.2016.05.014.

Mangiagalli, M. G., S. Marelli, and L. Guidobono Cavalchini. 2007. "Effect of Lycopene on Fowl Sperm Characteristics during in Vitro Storage." *Archiv Fur Geflugelkunde* 71 (1): 25–29.

Mara, L., S. Casu, A. Carta, and M. Dattena. 2013. "Cryobanking of Farm Animal Gametes and Embryos as a Means of Conserving Livestock Genetics." *Animal Reproduction Science* 138 (1–2): 25–38. doi:10.1016/j.anireprosci.2013.02.006.

Massip, A., S. Leibo, and E. Blesbois. 2004. "Cryobiology and the Breeding of Domestic Animals." In *Life in the Frozen State*, eds. Barry J. Fuller, Nick Lane and Erica E. Benson, 317–379. London: Taylor and Francis Group.

Miranda, M., B. Kulíková, J. Vašíček, L. Olexikova, N. Iaffaldano, and P. Chrenek. 2018. "Effect of Cryoprotectants and Thawing Temperatures on Chicken Sperm Quality." *Reproduction in Domestic Animals* 53 (1): 93–100. doi:10.1111/rda.13070.

Mitchell, R., and R. Buckland. 1976. "Fertility of Frozen Chicken Semen after Intravaginal and Intrauterine Inseminations Using Various Concentrations and Equilibration Times of Dimethylsulfoxide and a Range of Freezing and Thawing Rates." *Poultry Science* 55 (6): 2195–2200. doi:10.3382/ps.0552195.

Mocé, E., I. Grasseau, and E. Blesbois. 2010. "Cryoprotectant and Freezing-process Alter the Ability of Chicken Sperm to Acrosome React." *Animal Reproduction Science* 122 (3–4): 359–366. doi:10.1016/j.anireprosci.2010.10.010.

Mocé, E., P. H. Purdy, and J. K. Graham. 2010. "Treating Ram Sperm with Cholesterol-loaded Cyclodextrins Improves Cryosurvival." *Animal Reproduction Science* 118 (2–4): 236–247. doi:10.1016/j.anireprosci.2009.06.013.

Moghbeli, M., H. Kohram, A. Zare-Shahaneh, M. Zhandi, H. Sharideh, and M. Sharafi. 2016b. "Effect of Sperm Concentration on Characteristics and Fertilization Capacity of Rooster Sperm Frozen in the Presence of the Antioxidants Catalase and Vitamin E." *Theriogenology* 86 (6): 1393–1398. doi:10.1016/j.theriogenology.2016.03.038.

Moghbeli, M., H. Kohram, A. Zare-Shahaneh, M. Zhandi, M. Sharafi, M. M. Nabi, V. Zahedi, and H. Sharideh. 2016a. "Are the Optimum Levels of the Catalase and Vitamin E in Rooster Semen Extender after Freezing-thawing Influenced by Sperm Concentration?" *Cryobiology* 72 (3): 264–268. doi:10.1016/j.cryobiol.2016.03.008.

Mohan, J., S. Sharma, G. Kolluri, and K. Dhama. 2018. "History of Artificial Insemination in Poultry, Its Components and Significance." *World's Poultry Science Journal* 74 (3): 475–488. doi:10.1017/S0043933918000430.

Moore, S., and J. Hasler. 2017. "A 100-Year Review: Reproductive Technologies in Dairy Science." *Journal of Dairy Science* 100 (12): 10314–10331. doi:10.3168/jds.2017-13138.
Morrell, J., and I. Mayer. 2017. "Reproduction Biotechnologies in Germplasm Banking of Livestock Species: A Review." *Zygote* 25 (5): 545. doi:10.1017/S0967199417000442.
Mortimer, D. 2004. "Current and Future Concepts and Practices in Human Sperm Cryobanking." *Reproductive Biomedicine Online* 9 (2): 134–151. doi:10.1016/S1472-6483(10)62123-2.
Mosca, F., M. Madeddu, A. A. Sayed, L. Zaniboni, N. Iaffaldano, and S. Cerolini. 2016. "Combined Effect of Permeant and Non-permeant Cryoprotectants on the Quality of Frozen/thawed Chicken Sperm." *Cryobiology* 73 (3): 343–347. doi:10.1016/j.cryobiol.2016.10.001.
Motta, J. P. R., F. H. Paraguassú-Braga, L. F. Bouzas, and L. C. Porto. 2014. "Evaluation of Intracellular and Extracellular Trehalose as a Cryoprotectant of Stem Cells Obtained from Umbilical Cord Blood." *Cryobiology* 68 (3): 343–348. doi:10.1016/j.cryobiol.2014.04.007.
Mphaphathi, M. L., M. M. Seshoka, D. Luseba, B. Sutherland, and T. L. Nedambale. 2016. "The Characterisation and Cryopreservation of Venda Chicken Semen." *Asian Pacific Journal of Reproduction* 5 (2): 132–139. doi:10.1016/j.apjr.2016.01.009.
Nakamura, Y. 2016. "Poultry Genetic Resource Conservation Using Primordial Germ Cells." *Journal of Reproduction and Development* 62 (5): 431–437. doi:10.1262/jrd.2016-052.
Neville, W., J. Macpherson, and B. Reinhart. 1971. "The Contraceptive Action of Glycerol in Chickens." *Poultry Science* 50 (5): 1411–1415. doi:10.3382/ps.0501411.
Nguyen, T. M. D., S. Alves, I. Grasseau, S. Métayer-Coustard, C. Praud, P. Froment, and E. Blesbois. 2014. "Central Role of 5′-AMP-activated Protein Kinase in Chicken Sperm Functions." *Biology of Reproduction* 91 (5): 121, 121–115. doi:10.1095/biolreprod.114.121855.
O'Connell, M., N. Mcclure, and S. Lewis. 2002. "The Effects of Cryopreservation on Sperm Morphology, Motility and Mitochondrial Function." *Human Reproduction* 17 (3): 704–709. doi:10.1093/humrep/17.3.704.
Partyka, A., E. Łukaszewicz, and W. Niżański. 2012. "Lipid Peroxidation and Antioxidant Enzymes Activity in Avian Semen." *Animal Reproduction Science* 134 (3–4): 184–190. doi:10.1016/j.anireprosci.2012.07.007.
Partyka, A., D. Bonarska-Kujawa, M. Sporniak, M. Strojecki, and W. Niżański. 2016. "Modification of membrane cholesterol and its impact on frozen-thawed chicken sperm characteristics." *Zygote 24* (5): 714–723.
Partyka, A., M. Strojecki, and W. Niżański. 2018. "Cyclodextrins or Cholesterol-loaded-cyclodextrins? A Better Choice for Improved Cryosurvival of Chicken Spermatozoa." *Animal Reproduction Science* 193: 235–244. doi:10.1016/j.anireprosci.2018.04.076.
Partyka, A., O. Rodak, J. Bajzert, J. Kochan, and W. Niżański. 2017. "The Effect of L-carnitine, Hypotaurine, and Taurine Supplementation on the Quality of Cryopreserved Chicken Semen." *BioMed Research International* 2017: 1–8. doi:10.1155/2017/7279341.
Pérez-Marín, C. C., A. Arando, C. Mora, and A. Cabello. 2019. "Fertility after Insemination with Frozen-thawed Sperm Using N-methylacetamide Extender on the Combatiente Español Avian Breed." *Animal Reproduction Science* 208: 106111. doi:10.1016/j.anireprosci.2019.106111.
Pickett, B., and W. Berndtson. 1974. "Preservation of Bovine Spermatozoa by Freezing in Straws: A Review." *Journal of Dairy Science* 57 (11): 1287–1301. doi:10.3168/jds.S0022-0302(74)85058-7.
Pickett, B., W. Berndtson, and J. Sullivan. 1978. "Influence of Seminal Additives and Packaging Systems on Fertility of Frozen Bovine Spermatozoa." *Journal of Animal Science* 47 (Suppl 2): 12–47.
Polge, C. 1951. "Functional Survival of Fowl Spermatozoa after Freezing At– 79° C." *Nature* 167: 949–950. doi:10.1038/167949b0.
Polge, C., A. U. Smith, and A. S. Parkes. 1949. "Revival of Spermatozoa after Vitrification and Dehydration at Low Temperatures." *Nature* 164: 666. doi:10.1038/164666a0.
Purdy, P., and J. Graham. 2004. "Effect of Cholesterol-loaded Cyclodextrin on the Cryosurvival of Bull Sperm." *Cryobiology* 48 (1): 36–45. doi:10.1016/j.cryobiol.2003.12.001.

Purdy, P., Y. Song, F. Silversides, and H. Blackburn. 2009. "Evaluation of Glycerol Removal Techniques, Cryoprotectants, and Insemination Methods for Cryopreserving Rooster Sperm with Implications of Regeneration of Breed or Line or Both." *Poultry Science* 88 (10): 2184–2191. doi:10.3382/ps.2008-00402.

Qi, X.-L., K. Xing, Z. Huang, Y. Chen, L. Wang, L.-C. Zhang, X.-H. Sheng, X.-G. Wang, H.-M. Ni, and Y. Guo. 2020. "Comparative Transcriptome Analysis Digs Out Genes Related to Antifreeze between Fresh and Frozen–thawed Rooster Sperm." *Poultry Science* 99 (6): 2841–2851. doi:10.1016/j.psj.2020.01.022.

Quinn, J., and W. Burrows. 1936. "Artificial Insemination in Fowls." *Journal of Heredity* 27 (1): 31–38. doi:10.1093/oxfordjournals.jhered.a104138.

Rakha, B., M. Ansari, S. Akhter, I. Hussain, and E. Blesbois. 2016. "Cryopreservation of Indian Red Jungle Fowl (Gallus Gallus Murghi) Semen." *Animal Reproduction Science* 174: 45–55. doi:10.1016/j.anireprosci.2016.09.004.

Rakha, B., M. Ansari, S. Akhter, J. Santiago-Moreno, and E. Blesbois. 2018. "Cryoprotectant Effects of Egg Yolk on Indian Red Jungle Fowl (Gallus Gallus Murghi) Sperm." *Theriogenology* 119: 150–155. doi:10.1016/j.theriogenology.2018.06.015.

Rakha, B. A., M. S. Ansari, S. Akhter, Z. Zafar, I. Hussain, J. Santiago-Moreno, and E. Blesbois. 2017. "Cryopreservation of Indian Red Jungle Fowl (Gallus Gallus Murghi) Semen with Polyvinylpyrrolidone." *Cryobiology* 78: 27–33. doi:10.1016/j.cryobiol.2017.07.006.

Salehi, M., A. H. Mahdavi, M. Sharafi, and A. Shahverdi. 2020. "Cryopreservation of Rooster Semen: Evidence for the Epigenetic Modifications of Thawed Sperm." *Theriogenology* 142: 15–25. doi:10.1016/j.theriogenology.2019.09.030.

Salmani, H., M. M. Nabi, H. Vaseghi-Dodaran, M. B. Rahman, A. Mohammadi-Sangcheshmeh, M. Shakeri, A. Towhidi, A. Z. Shahneh, and M. Zhandi. 2013. "Effect of Glutathione in Soybean Lecithin-based Semen Extender on Goat Semen Quality after Freeze-thawing." *Small Ruminant Research* 112 (1–3): 123–127. doi:10.1016/j.smallrumres.2012.12.015.

Santiago-Moreno, J., C. Castaño, A. Toledano-Díaz, M. Coloma, A. López-Sebastián, M. Prieto, and J. Campo. 2011. "Semen Cryopreservation for the Creation of a Spanish Poultry Breeds Cryobank: Optimization of Freezing Rate and Equilibration Time." *Poultry Science* 90 (9): 2047–2053. doi:10.3382/ps.2011-01355.

Seigneurin, F., and E. Blesbois. 1995. "Effects of the Freezing Rate on Viability and Fertility of Frozen-thawed Fowl Spermatozoa." *Theriogenology* 43 (8): 1351–1358. doi:10.1016/0093-691X(95)00119-S.

Sexton, T. 1973. "Effect of Various Cryoprotective Agents on the Viability and Reproductive Efficiency of Chicken Spermatozoa." *Poultry Science* 52 (4): 1353–1357. doi:10.3382/ps.0521353.

Sexton, T. 1974. "Comparison of Various Cryoprotective Agents on Washed Chicken Spermatozoa: 4. Metabolism and Release of Glutamic-Oxalacetic Transaminase." *Poultry Science* 53 (1): 284–287. doi:10.3382/ps.0530284.

Sexton, T. 1979. "Effect of Prefreeze Procedure on the Fertility of Frozen Turkey Semen." *Poultry Science* 58 (4): 1106.

Sexton, T. 1980. "Optimal Rates for Cooling Chicken Semen From+ 5 To– 196 C." *Poultry Science* 59 (12): 2765–2770. doi:10.3382/ps.0592765.

Sexton, T. 1981. "Effect of Prefreeze Treatment on the Fertilizing Capacity of Frozen Chicken Semen." *Poultry Science* 60 (7): 1552–1557. doi:10.3382/ps.0601552.

Shaffner, C. S., E. Henderson, and C. Card. 1941. "Viability of Spermatozoa of the Chicken under Various Environmental Conditions." *Poultry Science* 20 (3): 259–265. doi:10.3382/ps.0200259.

Shahverdi, A., M. Sharafi, H. Gourabi, A. A. Yekta, V. Esmaeili, M. Sharbatoghli, E. Janzamin, M. Hajnasrollahi, and F. Mostafayi. 2015. "Fertility and Flow Cytometric Evaluations of Frozen-thawed Rooster Semen in Cryopreservation Medium Containing Low-density Lipoprotein." *Theriogenology* 83 (1): 78–85. doi:10.1016/j.theriogenology.2014.07.044.

Shanmugam, M., A. Vinoth, K. Rajaravindra, and U. Rajkumar. 2014. "Evaluation of Semen Quality in Roosters of Different Age during Hot Climatic Condition." *Animal Reproduction Science* 145 (1–2): 81–85. doi:10.1016/j.anireprosci.2013.12.015.

附錄四

# ÉLIMINATION DU GLYCÉROL DE LA SEMENCE DÉCONGELÉE DE COQ PAR CENTRIFUGATION DANS UN GEL COLLOÏDAL SYNTHÉTIQUE

**Lin Hsiu-Lien Herbie[1,2], Grasseau Isabelle[1], Mermillod Pascal[1], Blesbois Elisabeth[1], Vitorino Carvalho Anaïs[1]**

*[1]PRC, UMR085, INRAE, Université de Tours, 37380 Nouzilly, France*
*[2]Physiology Division, Livestock Research Institute, COA, 71246 Tainan, Taiwan*
Anais.Carvalho@inrae.fr

**RÉSUMÉ**

La cryoconservation des spermatozoïdes est une méthode cruciale dans la conservation de la biodiversité des espèces et des races, mais aussi pour assurer la restauration des lignées lors de catastrophes sanitaires. Actuellement, le glycérol est l'agent cryoprotecteur le plus utilisé pour la cryoconservation des spermatozoïdes de coq, étant le plus efficace dans le maintien des fonctions du spermatozoïde, mais il est associé à un taux de réussite faible voir une totale stérilité lors de l'insémination. Par conséquent, nous cherchons à développer une méthode simple d'élimination du glycérol par centrifugation dans un gel colloïdal synthétique, le Percoll®. Après collecte, la semence de coqs a été congelée en glycérol 11% en paillettes avec un congélateur programmable. Lors de la décongélation, la semence a été déposée sur une solution de sucrose (200 mM) en présence de Percoll® (Solution Sucrose-Percoll®, SSP) dans du PBS puis centrifugée 15 min. Afin de définir les meilleures conditions, différentes concentrations de SSP (40, 50, 60 ou 70%) ainsi que deux températures (4 ou 20°C) ont été testées. La concentration de glycérol et la mobilité individuelle des spermatozoïdes ont été évaluées après centrifugation respectivement par le kit Glycerol Assay et par Computer-Assisted Sperm Analysis system (CASA). Nos données montrent que toutes les concentrations de SSP réduisent la présence de glycérol à moins de 120 mM (soit une élimination de 94% du glycérol initial). La centrifugation à 20°C permet une bonne sélection des spermatozoïdes mobiles et progressifs, en particulier pour SSP 40 et 70%. Notre nouvelle méthode permet donc d'éliminer efficacement le glycérol des semences de coq cryopréservées tout en sélectionnant les spermatozoïdes présentant une bonne mobilité. Comparée à la méthode conventionnelle de dilutions successives, elle permet de gagner 44% de temps de traitement des échantillons lors de l'élimination du glycérol.

**ABSTRACT**

Sperm cryopreservation is a crucial method not only to conserve the biodiversity of species and breeds, but also to restore animal populations during emergent epidemics. Currently, glycerol is the most widely used cryoprotectant to freeze chicken sperm due to its effectiveness for maintaining sperm functions, but it is associated with a reduction of fertility or a total infertility during insemination. Therefore, we tried to develop a simple method to remove glycerol from frozen-thawed chicken semen with a centrifugation in a synthetic colloidal gel, the Percoll®. After collecting, chicken semen was frozen in a diluent of 11% glycerol in straws with a programmable freezer. Thawed semen was layered on the solution containing 200 mM sucrose and Percoll® (Solution Sucrose-Percoll®, SSP) in PBS and centrifuged 15 min after. Different concentrations of SSP (40, 50, 60 or 70%) combined with two different temperatures (4 or 20°C) were tested to determine the best conditions of SSP centrifugation. Glycerol concentration and sperm individual motility were evaluated respectively by Glycerol Assay kit and Computer-Assisted Sperm Analysis system (CASA). Our results showed that all concentrations of SSP efficiently reduce glycerol concentration to less than 120 mM (equal to remove 94% of glycerol from thawed semen). Centrifugation at 20°C allowed an efficient selection for motile and progressive sperm especially with SSP 40 and 70%. It indicated that our novel method can efficiently remove glycerol from post-thaw chicken semen with a positive selection of motile sperm. Compared to the conventional method of serial dilutions, it can save 44% of time of glycerol removal process.

*Quatorzièmes Journées de la Recherche Avicole et Palmipèdes à Foie Gras, Tours, 9 et 10 mars 2022*

## INTRODUCTION

La conservation des ressources génétiques est une stratégie cruciale pour protéger la biodiversité, restaurer des espèces ou races menacées et contrer les épidémies ou les accidents de reproduction (Morrell and Mayer, 2017; Svoradová *et al*., 2018). Trois types cellulaires sont actuellement disponibles pour la cryobanque chez les oiseaux : les spermatozoïdes, les cellules germinales primordiales et le tissu gonadique. Parmi toutes ces méthodes, la cryoconservation des spermatozoïdes reste encore l'approche la plus répandue car elle se base sur la collecte de grandes quantités de cellules, le respect du bien-être animal, et la possibilité d'un stockage long ainsi que d'échanges de ressources à longue distance (Çiftci and Aygün 2018; Thélie *et al*., 2019; Cardoso *et al*., 2020). Actuellement, le glycérol est le cryoprotecteur le plus utilisé pour congeler les spermatozoïdes de coq (Abouelezz *et al*., 2015 ; Lin *et al*., 2021). Il fournit une excellente protection des spermatozoïdes leur permettant de résister aux impacts négatifs du processus congélation/décongélation (Blesbois and Brillard, 2007; Blesbois *et al*., 2007). Cependant, il est associé à une baisse de la fertilité pouvant être totale et il doit donc être éliminé avant l'insémination (Seigneurin and Blesbois, 1995; Thélie *et al*., 2019). Néanmoins jusqu'à présent, le processus d'élimination du glycérol est compliqué et se base sur des équipements particuliers (dont une chambre froide). Par conséquent, nous cherchons à développer une nouvelle méthode simple d'élimination du glycérol de la semence de coq décongelée, se basant sur le Percoll®, un gel classiquement utilisé pour sélectionner les spermatozoïdes mobiles chez les mammifères mais également sur de la semence fraiche de coq (*Lin et al*., 2020).

## 1. MATERIELS ET METHODES

### 1.1. Animaux

18 coqs adultes (entre 32 et 42 semaines d'âge) de lignée T44 (*Gallus gallus domesticus*, Sasso, France) ont été utilisés comme donneurs de semence. Tous les coqs ont été hébergés en cages individuelles à l'unité expérimentale INRAe UE-PEAT (2018, https://doi.org/10.15454/1.5572326250887292E12), nourris avec un régime standard, de l'eau à volonté et élevés à 20°C et sous un cycle lumineux de 14h de lumière et 10h d'obscurité par jour. Ce protocole expérimental a été approuvé par le Ministère de l'Éducation Supérieure, de la Recherche et de l'Innovation (MESRI) et le comité d'éthique Val-de-Loire (N° APAFIS #4026-2016021015509521).

### 1.2. Collecte, congélation et décongélation de la semence

La semence de coq a été collecté régulièrement deux fois par semaine par la méthode de massage abdominal (Burrows et Quinn, 1937). Trois groupes de 6 animaux ont été formés de façon aléatoire pour regrouper les semences dans un volume de Lake PC similaire à celui de collecte puis mélangés doucement et refroidis à 4°C pendant 10 min. Un volume égal de Lake PC contenant 22% de glycérol a été ajouté pour atteindre une concentration finale de 11% de glycérol, puis l'échantillon a été équilibré à 4°C avec une agitation douce pendant 10 min. Par la suite, la semence a été déposée dans des paillettes de 0,5 mL (IMV Technologies, France). Toutes les paillettes ont été scellées, transférées dans un congélateur programmable (MiniDigitcool, IMV Technologies, France) et congelées à vitesse contrôlée de 4 à -35°C à -7 °C/min puis de -35 à -140°C à -20 °C/min. Finalement, les paillettes ont été plongées dans de l'azote liquide pour une conservation d'au moins 14 jours et décongelées au bain-marie à 4°C pendant 3 min pour la suite des expériences.

### 1.3. Préparation de Solution Sucrose-Percoll® et élimination du glycérol

La solution de Sucrose-Percoll® (SSP) a été préparée en ajoutant du Percoll® (Sigma-Aldrich, USA) isotonique et du sucrose 200 mM pour composer 40, 50, 60 ou 70% de SSP dans du PBS, puis 2 mL de SSP ont été transférés dans un tube conique de 15 mL. La semence décongelée (5 répliques) a été déposée sur 2 mL de SSP et centrifugée 15 min à 800×g à 4 ou 20°C. Le culot de spermatozoïdes a été collecté et remis en suspension avec le dilueur Lake 7.1. Les témoins étaient la semence décongelée sans élimination du glycérol.

### 1.4. Évaluation de la concentration de glycérol et de la mobilité individuelle des spermatozoïdes

La concentration de glycérol a été mesurée selon les instructions du kit Glycerol Assay (Sigma-Aldrich, USA) sur 10 µL d'échantillon. La mobilité individuelle des spermatozoïdes a été mesurée au Computer-Assisted Sperm Analysis system (CASA, IVOS, IMV Technologies, France) sur 2 µL d'échantillon à $30 \times 10^6$ cellules/mL. Quatre champs d'observation ont été capturés pour chaque échantillon. La trajectoire de chaque spermatozoïde a été enregistrée afin de calculer sa vitesse moyenne (velocity average path, VAP). Les résultats de mobilité ont été exprimés en pourcentage de spermatozoïdes mobiles et progressifs qui ont présenté respectivement une VAP > 5 mm/s et VAP > 50 mm/s.

### 1.5. Analyses statistiques

Toutes les analyses statistiques sont réalisées avec le logiciel R (version 1.4.1717) par ANOVA à deux facteurs. Les résultats sont exprimés en moyennes et écart-type. Une différence significative est révélée par une p-valeur inférieure à 0,05%.

## 2. RESULTATS ET DISCUSSION

### 2.1. Concentration de glycérol après centrifugation

Nos données révèlent une réduction significative de la concentration de glycérol après traitement de la semence par une solution SSP (quelque soit la concentration de Percoll)(Tableau 1). En effet, alors que la concentration moyenne de glycérol de la semence décongelée est de 1866,3 mM, après centrifugation dans SSP, elle est réduite à moins de 120 mM dans tous les traitements (Tableau 2). Ces résultats révèlent que notre nouvelle méthode d'élimination du glycérol peut éliminer au moins 93,6% du glycérol de la semence décongelée. Des expériences antérieures indiquent que 2% de glycérol (soit environ 273.4 mM) est le seuil maximal pour obtenir la capacité maximale de fécondation (Polge, 1951; Neville *et al*., 1971). Ainsi, notre méthode permet d'obtenir une concentration de glycérol compatible avec de bonnes conditions d'insémination.

### 2.2. Mobilité des spermatozoïdes après centrifugation

La mobilité des spermatozoïdes décongelés est un indicateur de leur capacité fécondante chez les poulets (Blesbois *et al*., 2008). C'est pourquoi nous avons choisi d'étudier ce critère comme prédicteur potentiel de la fertilité. La concentration de SSP, la température mais également l'interaction de ces deux facteurs ont un impact significatif sur la mobilité des spermatozoïdes (Tableau 1). La mobilité des spermatozoïdes dans l'échantillon témoin diminue de façon importante à 20°C mais pas à 4°C dans les 15 min suivant la décongélation (Tableau 2). L'élimination du glycérol par notre protocole à 4°C ne montre aucune différence significative de la mobilité des spermatozoïdes. Cependant, le même protocole à 20°C améliore la mobilité des spermatozoïdes, en particulier dans le traitement SSP 70% qui maintient le pourcentage de spermatozoïdes mobiles et progressifs à respectivement de 25,1% et 15,2% plus élevés que le témoin. Cette observation est en accord avec celle précédemment rapportée par Lin *et al*., (2020) qui signalent que la centrifugation de semence sur une solution de Percoll® est bénéfique pour sélectionner les spermatozoïdes mobiles d'une semence fraîche de coq. En outre, un protocole basé sur un autre gel colloïdal, le gel Accudenz (AD), est déjà utilisé pour éliminer le glycérol de la semence décongelée de coq par centrifugation dans la solution AD discontinue (12% sur 30%) à 5°C (Purdy *et al*., 2009). Cependant, nos données suggèrent une meilleure mobilité des spermatozoïdes. La comparaison des deux protocoles dans les mêmes conditions permettrait d'identifier la méthode plus efficace.
De plus, les taux de mobilité observés à 20°C après centrifugation en SSP sont équivalents à ceux obtenus à 4°C, suggérant que ce protocole peut facilement être développé à température ambiante.

### 2.3. Durée du protocole d'élimination du glycérol

Actuellement, la centrifugation avec dilutions en série (CDS) est la méthode la plus courante pour réduire la concentration de glycérol de la semence décongelée de coq (Blesbois and Brillard, 2007). Toutefois, cette méthode nécessite de travailler pendant de long temps (Tableau 3). De plus, cette méthode ainsi que celle précédemment présentée basée sur le gel AD nécessite de travailler à 4°C, et donc la présence d'une chambre froide, ce qui est difficile à mettre en pratique dans les élevages. Au contraire, notre nouvelle méthode d'élimination du glycérol n'a pas besoin de la chambre froide et la centrifugation ne prend que 15 min, ce qui permet gagner respectivement 40% et 44,4% de temps par rapport à la méthode d'AD et de CDS.

## CONCLUSIONS

Nos données montrent l'efficacité d'une nouvelle méthode d'élimination du glycérol de la semence décongelée de coq basée sur le gel Percoll®. Cette méthode permet d'éliminer efficacement le glycérol de la semence décongelée tout en sélectionnant les spermatozoïdes présentant une meilleure mobilité. Par rapport à la méthode CDS conventionnelle ou celle basée sur le gel AD, elle est plus économique, à la fois en temps et en équipement car elle peut être employée à température ambiante et éviter donc la nécessité d'une chambre froide. Actuellement, des expériences sont en cours pour déterminer l'impact de cette méthode sur la réussite de l'insémination artificielle.

## REMERCIEMENTS

Les auteurs tiennent à remercier Philippe Didier, Joël Delaveau et Jérémy Bernard (INRAe UE-PEAT) pour leur investissement dans la gestion des animaux. Ce projet est financé par le projet [ANR-11- INBS-0003].

## REFERENCES BIBLIOGRAPHIQUES

Abouelezz F. Castaño C. Toledano-Díaz A. Esteso M. López-Sebastián A. Campo J. and Santiago-Moreno J., 2015. Theriogenology, (83), 1541–1547.
Blesbois E. and Brillard J. P., 2007. Animal, (1), 1472-1481.
Blesbois E., 2007. World's Poult. Sci. J., (63), 213-222.
Blesbois E. Grasseau I. Seigneurin F. Mignon-Grasteau S. Saint Jalme M. and Mialon M. M., 2008. Theriogenology, (69), 252–261.

Burrows W. and Quinn J., 1937. Poult. Sci., (16), 19-24.
Cardoso B. Sánchez-Ajofrín I. Castaño C. García-Álvarez O. Esteso M. C. Maroto-Morales A. Iniesta-Cuerda M. Garde J. J. Santiago-Moreno J. and Soler A. J., 2020. Animals, (10), 691., doi: 10.3390/ani10040691.
Çiftci H. and Aygün A., 2018. World's Poult. Sci. J., (74), 699-710.
Lin H. Chen Y. Lin D. Lai Y. Wu M. and Chen L., 2020. Br. Poult. Sci., (61), 86-91.
Lin H. H. Blesbois E. and Vitorino Carvalho A., 2021. World's Poult. Sci. J., doi: 10.1080/00439339.2022.1998816
Moore S. and Hasler J., 2017. J. of Dairy Sci., (100), 10314-10331.
Neville W. Macpherson J. and Reinhart B., 1971. Poult. Sci., (50), 1411-1415.
Polge C., 1951. Nature, (167), 949-950.
Purdy P. Song Y. Silversides F. and Blackburn H., 2009. Poult. Sci., (88), 2184-2191.
Seigneurin F. and Blesbois E., 1995. Theriogenology, (43), 1351-1358.
Svoradová A. Kuželová L. Vašíček J. Olexíková L. and Chrenek P., 2018. Biotechnol. Prog., (34), 778-783.
Thélie A. Bailliard A. Seigneurin F. Zerjal T. Tixier-Boichard M. and Blesbois E., 2019. Poult. Sci., (98), 447-455.

**Tableau 1.** Effets de la température et la concentration de Solution Sucrose-Percoll® sur la concentration de glycérol, le pourcentage des spermatozoïdes mobiles et progressifs

| | Statistique (p-valeur) | | |
|---|---|---|---|
| | **Température** | **Concentration de SSP** | **Température* Concentration de SSP** |
| **Concentration de glycérol** | 0.633 | <0,001 | 0.460 |
| **Spermatozoïdes mobiles** | <0,001 | <0,001 | <0,001 |
| **Spermatozoïdes progressifs** | <0,001 | <0,001 | 0.002 |

SSP : Solution Sucrose-Percoll®. Une p-valeur inférieure à 0,05 est considérée comme significative.

**Tableau 2.** Effets sur la concentration de glycérol, le pourcentage des spermatozoïdes mobiles et progressifs de la semence décongelée de coq après centrifugation dans Solution Sucrose-Percoll®

| | 4°C | | | | | 20°C | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **TEM** | **SSP 40%** | **SSP 50%** | **SSP 60%** | **SSP 70%** | **TEM** | **SSP 40%** | **SSP 50%** | **SSP 60%** | **SSP 70%** |
| **Concentration de glycérol (mM)** | 1885,1 ± 73,1[a] | 80.8 ± 10,5[b] | 79.1 ± 16,7[b] | 85.0 ± 17,9[b] | 118.8 ± 12,9[b] | 1847.5 ± 80,5[a] | 74.4 ± 20,3[b] | 76.8 ± 22,7[b] | 112.5 ± 19,5[b] | 111.9 ± 21,5[b] |
| **Spermatozoïdes mobiles (%)** | 44.0 ± 3,9[ab] | 46.1 ± 3,7[ab] | 43.1 ± 5,2[ab] | 42.5 ± 4,6[ab] | 46.4 ± 3,6[ab] | 25.4 ± 3,3[c] | 42.5 ± 3,4[ab] | 39.9 ± 4,4[a] | 40.1 ± 3,7[a] | 50.5 ± 5,5[b] |
| **Spermatozoïdes progressifs (%)** | 28.3 ± 2,0[ab] | 31.6 ± 3,1[a] | 28.5 ± 3,7[ab] | 27.6 ± 3,5[ab] | 30.6 ± 2,9[ab] | 16.5 ± 3,7[c] | 26.4 ± 2,6[ab] | 24.1 ± 3,5[b] | 24.9 ± 2,5[ab] | 31.7 ± 4,9[a] |

TEM : échantillon témoin ; SSP : Solution Sucrose-Percoll® ; Les valeurs sont exprimées en moyenne ± écart-type (n = 5) ; [abc] Des exposants différents indiquent des différences significatives (p-valeur<0,05).

**Tableau 3.** Comparaisons des méthodes d'élimination du glycérol entre la centrifugation par Sucrose-Percoll® solution, Accudenz et avec dilutions en série

| | Solution | Dilution | | Centrifugation | | |
|---|---|---|---|---|---|---|
| | | T (°C) | Time (min) | Force (g) | T (°C) | Time (min) |
| SPS | Sucrose-Percoll® | - | 0 | 800 | 4 or 20 | 15 |
| AD | Accudenz discontinu | - | 0 | 300 | 5 | 25 |
| CDS | Lake C | 4 | 12 | 500 | 4 | 15 |

SSP : Solution Sucrose-Percoll® ; AD : Accudenz ; CDS : centrifugation avec dilutions en série.

Video link: https://www.inrae.fr/actualites/videos-trois-doctorants-temoignent-leurs-travaux-recherche

附錄六

INRAE

# Exploring how glycerol impacts fertility capacity of chicken sperm

Hsiu-Lien Herbie Lin[1,2], Isabelle Grasseau[1], Pascal Mermillod[1], Elisabeth Blesbois[1], Anaïs Vitorino Carvalho[1]

[1] INRAE, CNRS, IFCE, Université de Tours, PRC, 37380, Nouzilly, France; [2] Physiology Division, Livestock Research Institute (LRI), COA, 71246, Tainan, Taiwan

26ème Journée Thésards de l'unité PRC INRAE Val de Loire – 15 et 16 Juin 2022

## INTRODUCTION

Glycerol is an excellent cryoprotectant for vertebrate sperm against cryodamages[1]. In chicken, 11% glycerol is the standard concentration for freezing sperm, but it needs to be eliminated before inseminating hens to avoid infertility[2]. This phenomenon has been discussed since 1950s[3], but the mechanisms involved in negative glycerol effects are still unknown. Here we hypothesized that glycerol disturb sperm biology before (during freezing and thawing processes) and after (in the female tract) insemination. Thus, we firstly confirmed the effect of increasing glycerol concentrations on fertility. Then we investigated how sperm biology was impacted by glycerol presence at 4 and 41°C within 60 min (time necessary for cryopreservation, thawing, insemination and progression in the female tract).

## METHODS

1. Semen of 10 adult T44 roosters was collected, pooled, and diluted with Lake PC diluent combined to 0, 1, 2, 6 and 11% glycerol at 4°C.
2. For fertility test (n=2), 80 Isabrown hens were inseminated immediately after dilution with $100 \times 10^6$ pooled glycerolized sperm/female.
3. For *in vitro* tests (n=5), sperm membrane integrity, mitochondria activity, apoptosis, ROS balance and ATP content were assessed at 0, 30 and 60 min at 4 and 41°C with fluorescent probes and ATP-luciferase reaction kit by flow cytometry or luminometer.
4. Data of fertility and *in vitro* sperm biology were analyzed by Chi2 test and two-way ANOVA with Rstudio and GraphPad softwares, respectively. Significant difference was identified by different letters ($p<0.05$).

## RESULT-1 Impacts of glycerol on fertility

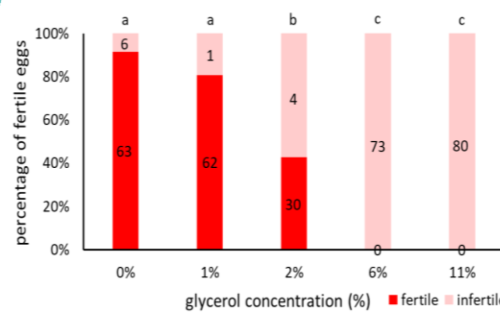



**Figure 1.** Effects of glycerol on hen fertility. Red bars represent the percentage of fertile eggs (%). The numbers on the bars represent the eggs numbers.

2% glycerol in semen caused more than 50% loss of fertility and 6% led to a complete infertility.

## CONCLUSIONS and PERSPECTIVES

1. Glycerol causes distinguishable impacts on sperm motility and biology at 41°C, which might probably alter normal functions of sperm progression, selection and storage in the female tract, resulting in decreased fertility.
2. ROS balance and ATP content are two major factors regulating sperm motility. Our future *in vitro* studies will narrow down to verify their connections.
3. Further *in vivo* and *ex vivo* experiments will be applied to decipher the deleterious effects of glycerol during sperm progression or storage in the female tract.

## RESULT-2 Impacts of glycerol on sperm biology

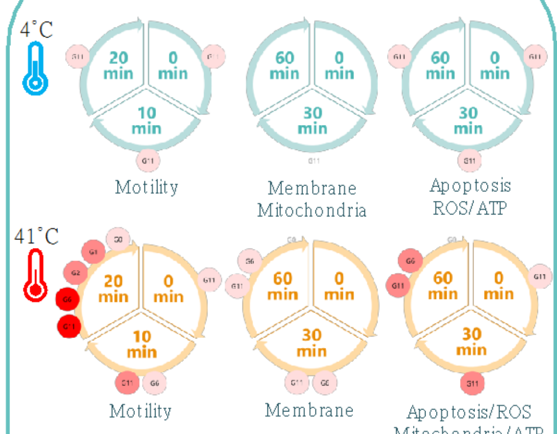



**Figure 2.** Effects of glycerol concentration on sperm biology. G0, 1, 2, 6, 11 represent glycerol 0, 1, 2, 6, 11%. ● ● ● represent mild, medium and severe impacts.

1. At 4°C, only 11% glycerol caused mild impacts on sperm biology.
2. At 41°C, glycerol severely impaired sperm motility in 10-20 min.
3. Glycerol induced sperm metabolism modifications mainly occurred when concentration higher than 6%.

## REFERENCES

1. Abouelezz et al. 2015. Theriogenology 83 (9):1541-1547.
2. Seigneurin and Blesbois. 1995. Theriogenology 43 (8):1351-1358.
3. Polge, 1951. Nature (167) 949-950.

### W155
### RECOMBINANT PORCINE JUNO BEADS SUPPORT SPECIES-SPECIFIC SPERM BINDING

*G. Yarza-Muñoz [1], R. Romar [2], M. Jiménez-Movilla [1]*
*[1]Department of Cell Biology and Histology, School of Medicine, University of Murcia, Campus Mare Nostrum and IMIB-Arrixaca, Murcia, Spain.*
*[2]Department of Physiology, Faculty of Veterinary, University of Murcia, Campus Mare Nostrum and IMIB-Arrixaca, Murcia, Spain.*

BACKGROUND-AIM
Juno protein is localized on the egg's membrane and it is essential in mammals fertilization due its binding to the sperm receptor IZUMO1. Knock out for some of these two proteins show absence of fertilization. So, we propose the use of beads coated with recombinant JUNO protein to bind those spermatozoa with the greatest capacity to fertilise.
METHODS
We used two plasmid vectors pcDNA3.1 to cloning the predicted sequence encoding JUNO gene with a 6-histidine tag added to the C-terminus, from Sus scrofa and Homo sapiens. Human Embryonic Kidney (HEK) 293T cells were transiently transfected with the plasmid vectors to produce the recombinant porcine and human JUNO protein (rpJUNO and rhJUNO). Secreted proteins were purified and conjugated to commercial magnetic beads (His Mag Sepharose Excel GE Healthcare) to generate the 3D models pJUNO-beads and hJUNO-beads. 3D models were co-incubated for 1, 2, 3 and 4 hours with fresh ejaculated boar semen from fertile males to assess the number of spermatozoa bound per bead and the number of bound spermatozoa reacted acrosome. Beads without protein (Ctrl-beads) were used as internal control for the assay. Beads were fixed in glutaraldehyde and stained with Hoechst and PNA-FITC. We studied a total of 605 beads and statistical analysis was performed by one-way ANOVA and when P-value was $<0.05$, differences among groups were analysed by a Tukey's.
RESULTS
Expression of proteins and its conjugation to commercial beads was confirmed by SDS-PAGE and Western-Blot. The greatest significant differences in the number of spermatozoa bound were observed at 4 hours (P= 0) with 12,58±1,38 for pJUNO-beads, 7,96±0,98 for hJUNO-beads and 5,69±0,61 for Ctrl-beads. The number of reacted spermatozoa at 4 hours was significative higher (P= 0) in pJUNO-beads with 5,68±0,67 while in human and control models had 3,43±0,39 and 2,64±0,23 respectively.
CONCLUSIONS
These preliminary data show that JUNO proteins were successfully expressed and secreted by HEK cells and conjugated to commercial beads. The generated pJUNO-beads model binds more reacted sperms than control and human models showing that porcine sperm specifically recognize porcine JUNO protein hence, indicating that the Juno-sperm binding is species-specific.
Supported: Fundación Séneca 20887/PI/18; Ministerio de Ciencia e Innovación PID2020-114109GB-I00

### W156
### IMPACT OF GLYCEROL ON SPERM FERTILIZING CAPACITY IN CHICKEN

*H.H. Lin [1], I. Grasseau [1], P. Mermillod [1], E. Blesbois [1], A. Vitorino Carvalho [1]*
*[1]INRAE, CNRS, IFCE, Université de Tours, PRC, Nouzilly, France*

BACKGROUND-AIM
Glycerol is the most common cryoprotectant of vertebrate semen because it provides an excellent protection of sperm against cryodamages. In chicken, 11% glycerol is the standard concentration for freezing sperm, but it needs to be removed before inseminating hens, otherwise the fertility is dramatically decreased. This phenomenon has been discussed since 1950s, but the mechanisms involved remain unclear. Here we hypothesize that glycerol preserves sperm capacities at low temperature whereas interfere with sperm biology at physiological temperature (i.e. 41°C). Thus, we firstly investigated the effect of increasing glycerol concentrations on fertility. Secondly, we explored how sperm biology, especially sperm motility and membrane integrity, could be impaired by glycerol presence at 41°C within 60 min (physiological time of sperm evolving from vagina to infundibulum).
METHODS
Semen of 10 adult T44 roosters was collected, pooled, and diluted with glycerol-Lake PC diluent to final concentration of 0, 1, 2, 6 and 11% glycerol at 4°C. For fertility test (n=2), 80 Isabrown hens were inseminated immediately after dilution with 100×106 pooled glycerolized sperm/female. For in vitro tests (n=5), sperm mass and individual motilities were assessed at 0, 10, 20 and 30 min at 41°C by microscope and computer-assisted sperm analysis (CASA), respectively, and membrane integrity was evaluated at 0, 30 and 60 min by fluorescence flow cytometry. Data were analysed by ANOVA tests.
RESULTS
Results revealed that 2% glycerol led to 50% decline of fertility, and infertility was observed with 6 and 11% glycerol. No impact of glycerol on sperm mass motility was revealed but 1% glycerol significantly reduced sperm individual motility after 10 min, and more severe reduction was observed with higher concentrations. Moreover, whereas no impact was observed with 1 and 2% glycerol, 6 and 11% glycerol induced sperm membrane integrity failure after 30 min of incubation at 41°C.
CONCLUSIONS
Collectively, the presence of glycerol in semen samples at very low concentration impacted fertility and sperm motility, whereas higher concentrations are also associated with membrane defects, supporting the need to remove glycerol before insemination to stop the disturbances of sperm transportation and integrity in female tract.

附錄八

*26th World's Poultry Congress, abstracts selected in 2022*

**ID: 1939**

# APPLICATION OF COLLOIDAL CENTRIFUGATION TO REMOVE GLYCEROL FROM CHICKEN FROZEN-THAWED SEMEN

H. Lin*(1), I. Grasseau (1), P. Mermillod (1), E. Blesbois (1), A. Vitorino Carvalho (1)

(1) INRAe, Nouzilly, France

**Presenting author*

**Corresponding author mail:** linherbie@gmail.com (Hsiu-Lien Herbie Lin)

Glycerol is the most used cryoprotectant to freeze chicken sperm due to an excellent protection of sperm cells against cryodamage. However, the presence of glycerol in post-thawed semen causes severe fertility reduction, leading to the necessity of removing glycerol before insemination. The main approach developed to remove glycerol is serial dilution (SD) protocol, which implies special equipment to maintain the thawed semen at 4°C and is time consuming. Therefore, we developed a simple method to remove glycerol from chicken frozen-thawed semen based on centrifugation in a colloidal gel, Percoll®, which is ordinarily used to select motile sperm in mammals as well as in fresh chicken semen. Eighteen adult T44 roosters were randomly divided between three groups and used as semen donors. Semen was collected from each group and frozen in Lake PC diluent containing 11% glycerol in straws in a programmable freezer. Frozen semen was thawed at 4°C for 3 min, layered on PBS solution containing 200 mM sucrose and isotonic Percoll® (Sucrose-Percoll® Solution, SPS) and centrifuged at 20°C, 800×g for 15 min. Different concentrations of SPS in PBS (40, 50, 60 or 70%) were tested to identify the best conditions by performing in vitro tests including glycerol concentration assay and sperm individual motility evaluation, as indicators of in vivo fertility. Artificial inseminations were performed on 36 hens with frozen-thawed semen treated by SPS and by SD protocols to compare their efficiencies. Data of in vitro and fertility tests were analysed by two-way ANOVA and Chi2 tests. Our results indicated that all concentrations of SPS efficiently decreased glycerol concentration to less than 120 mM while the concentration was 1866.3 mM before treatment. A beneficial selection of motile and progressive sperm was achieved by all concentrations of SPS, especially SPS 70% showed the best improvement of 25,1% and 15,2%. SPS 40 and 70% were two conditions selected for insemination test and reached 45.8% and 21.6% of fertility respectively, compared to 49.1% after SD treatment. Collectively, our new colloidal method can efficiently remove glycerol from chicken frozen-thawed semen with a positive selection of sperm motility. SPS 40% obtained better fertility than SPS 70% but was not significantly different from SD. Compared to the conventional SD protocol, this method can save 44% of the time while achieving similar fertility.

附錄九

**Session 6: 4:15pm March 9th**

**Impacts of glycerol on sperm fertilizing ability in chickens**

Hsiu-Lien Herbie Lin*, Isabelle Grasseau, Pascal Mermillod, Elisabeth Blesbois, Anaïs Vitorino Carvalho

*INRAE, Nouzilly, France

Abstract:

Glycerol is the most efficient cryoprotectant to freeze chicken sperm, but it is associated with fertilization failures, requiring its removal before insemination. The cellular mechanisms underlying these negative effects on fertility remain unknown. Here, we firstly investigated the impacts of glycerol (0,1, 2, 6 and 11%) on sperm biology within 60 min of incubation at two temperatures to mimic freezing procedures (4°C) and physiological temperature (41°C). After collection, semen of 10 roosters was pooled, incubated with different doses of glycerol and sperm motility, membrane integrity, apoptosis, mitochondria activity and ATP production were assessed. Secondly, we explored how glycerol modulates sperm storage in oviduct. Nine hens were inseminated with Hoechst stained sperm (200×106 sperm/female), slaughtered after 24 hours, and the presence of sperm was explored in sperm storage tubules (SST) at uterovaginal junction. 11% glycerol is the only concentration interfering with sperm biology at 4°C whereas 6 and 11% glycerol significantly impair sperm characteristics at 41°C, especially in terms of motility. Furthermore, % of SST containing sperm decreased with increasing glycerol concentrations. Collectively, our data revealed important impacts of glycerol on sperm motility, potentially responsible of the reduction of sperm storage in SST observed in vivo after insemination, explaining reduced fertility.

Keywords: chicken, sperm, glycerol l