Università degli Studi del Molise

"*Department of Agricultural, Environmental and Food Sciences*"

International PhD Course in:
Agricultural Technologies and Biotechnologies Curriculum: Welfare, Biotechnology and Quality of Animal Production (XXXVI Cycle)
Related Disciplinary Scientific Sector: AGR/20 (Zooculture)

PhD thesis

"Optimizing genetic resource conservation and semen cryobanking in aquatic and avian species with a focus on investigating the impact of ovarian fluid onfertilization success in native Mediterranean trout"

Ph.D. Candidate: **Emanuele Antenucci**

Coordinator of Ph.D. Course: Prof. Giuseppe Maiorano
Supervisor: Prof.ssa Nicolaia Iaffaldano

UNIVERSITY OF MOLISE

Department of Agricultural, Environmental and Food Sciences

International PhD Course in:

Agricultural Technologies and Biotechnologies

Curriculum: Welfare, Biotechnology and Quality of Animal Production

(XXXVI Cycle)

Related Disciplinary Scientific Sector: AGR/20 (Zooculture)

PhD thesis

"Optimizing genetic resource conservation and semen cryobanking in aquatic and avian species with a focus on investigating the impact of ovarian fluid on fertilization success in native Mediterranean trout"

Coordinator of Ph.D. Course: *Prof. Giuseppe Maiorano*

Supervisor: *Prof.ssa Nicolaia Iaffaldano*

Ph.D. Candidate: **Emanuele Antenucci**

169959

Academic year 2022/2023

“Ma l'impresa eccezionale,
dammi retta, è essere normale.”

LUCIO DALLA

ABSTRACT **1**

RIASSUNTO **4**

GENERAL INTRODUCTION *7*

Part 1. ***Animal Biodiversity: Definition, Primary Threats, and Current State of Resources*** ***8***

1. Conservation of Biodiversity **8**

1.1 Definition and Levels of Biodiversity *8*

1.2 Causes of biodiversity loss *9*

1.4 Status and trends of animal genetic resources *12*

1.5 Biodiversity in the livestock sector *14*

1.5.1 The relevance of local native breeds *15*

2. Fish Biodiversity **18**

2.1 The state of the fishery resources: in particular the Mediterranean brown trout *19*

3. Poultry Biodiversity: Importance and main threats **23**

3.1 Poultry Genetic Resource in Italy *25*

Part 2. ***Overview of animal genetic diversity conservation with a special emphasis on ex situ in vitro conservation strategy*** ***28***

4. Animal biodiversity conservation strategies **28**

4.1 Ex situ cryopreservation of germplasm *29*

4.1.1 Cryopreservation methods *29*

4.1.2 Advantages and disadvantages of sperm cryopreservation *30*

4.1.3 Cryopreservation of oocytes and embryos *31*

4.14 Cryopreservation of Somatic Cells and Stem cell *31*

4.1.5 Other germoplasm *32*

4.2 Cryobank: Preservation of Animal Species with Ex Situ in Vitro Technology *33*

Part 3. ***Notes on the spermatozoa of fish and poultry*** ***36***

5. Specific feature of teleost fish semen **36**

5.1 Female Gametes, Ovarian Fluid and Effects of Ovarian Fluid on the Performance of Freshwater Fish Sperm *38*

6. Specific characteristics of poultry semen **44**

Part 4. Safeguarding the animal biodiversity: LIFE Nat.Sal.Mo project and TuBAvI-2 ..... **48**

7. Recovery of S. Macrostigma: Application of Innovative Techniques and Participatory Governance Tools in rivers of Molise - LIFE Nat.Sal.Mo **48**

7.1 Selective access to spawning ground by fixed traps *51*

7.2 Semen cryobank *51*

7.3 Artificial nesting *55*

8. Biodiversity conservation in Italian poultry breeds: TuBAvI-2 ..... **56**

REFERENCES **59**

Session 1. Improving fertilisation success: insights from ovarian fluid studies in Mediterranean trout (*S. Cetti*) 76

PREFACE 77

STUDY 1. The Use of Ovarian Fluid as Natural Fertilization Medium for Cryopreserved Semen in Mediterranean Brown Trout: The Effects on Sperm Swimming Performance 82

1. Introduction 84

2. Materials and Methods 85

3. Results 87

4. Discussion 90

5. Conclusions 93

STUDY 2. Zootechnical Brown Trout (*Salmo trutta* L. 1758) Ovarian Fluid Fails to Upregulate the Swimming Performances of Native Mediterranean Brown Trout (*Salmo cettii* Rafinesque, 1810) Sperm in the Biferno River 98

1.Introduction 100

2. Materials and Methods 102

3. Results 105

4. Discussion 108

5. Conclusions 109

Session 2. Preserving Avian Heritage: Establishing Italy's First Semen Cryobank for Native Chicken and Turkey Breeds 115

PREFACE 116

STUDY 1. Italian semen cryobank of autochthonous chicken and turkey breeds: a tool for preserving genetic biodiversity 120

1. Introduction 122

2. Materials and methods 129

3. Results 130

4. Discussion 132

5. Conclusions 135

STUDY 2. The Effect of Semen Cryopreservation Process on Metabolomic Profiles of Turkey Sperm as Assessed by NMR Analysis 143

1. Introduction 145

2. Materials and Methods 147

3. Results 151

4. Discussion 154

5.Conclusions 160

CONCLUSIONS AND FUTURE PERSPECTIVES 169

LIST OF PUBLICATIONS 173

ABSTRACT

In Italy, over the years, there has been a progressive decrease in animal biodiversity, mainly due to the combined effect of harmful human actions, continuous loss and fragmentation of habitats, pollution, and climate change. In this regard, the preservation of animal biodiversity has become increasingly important over the years, not only from the perspective of livestock breeding, which benefits from the preservation of different species, but also for animals living in the wild environment. Their survival is crucial to protect and recover the precious natural heritage at the national and international levels, through the maintenance of high genetic variability able to ensure greater ecological stability, enabling populations to adapt to climate and environmental changes.

According to the Food and Agriculture Organization (FAO) of the United Nations, the biodiversity conservation can be achieved through the use of various techniques such as *in situ* and *ex situ*. The *ex situ* technique can be further divided into *in vivo* and *in vitro*. The *in situ* technique involves the maintenance of live animals within the livestock production system in which they were developed, while in the *ex situ in vivo* technique, the maintenance of live animals takes place outside their original habitat. The *ex situ in vitro* technique requires cryopreservation of genetic material in haploid form (sperm and oocyte), diploid form (embryos, somatic cells), or DNA sequences (FAO 2012, 2015). In the last two decades, at the European level, safeguarding animal genetic resources has played a central role in important conservation programmes that envisaged the adoption of both *in situ* and *ex situ* strategies. Among these, of particular interest is our European LIFE project entitled *'Recovery of S. Macrostigma: Application of Innovative Techniques and Participatory Governance Tools in the rivers of Molise'* (Acronym Nat.Sal.Mo). *Salmo Macrostigma* syn. *Salmo cettii* is an endemic species of the Mediterranean area, currently classified as 'vulnerable' in Europe and 'endangered' in Italy.

The main threats to the species include genetic introgression through crossbreeding with zootechnical strains, loss of natural habitats, and unregulated fishing. The primary objective of the project is to ensure the recovery and conservation of native Mediterranean trout in the basins of the Biferno and Volturno rivers (Molise region), using two innovative techniques: sperm freezing with relative creation of the first European cryobank, and the installation of semi-natural nests. In the Nat.Sal.Mo project, the use of doses of frozen semen from the cryobank, combined with appropriate fertilization schemes, has proven to be an effective strategy for restoring the genetic integrity of this species, allowing the maintenance of a high genetic variability in offspring and population fitness. Another innovative technique involves the construction of semi-natural nests, which emulate the behaviour of females in nature. This approach ensures greater success by minimizing non-adaptive selective pressure, as eggs are seeded close to hatching, avoiding exposure of larvae and early life stages to an artificial environment.

In Italy, even the drastic decrease in native poultry breeds constitutes an emergency, mainly resulting from the widespread use of high-performance hybrids in the national poultry sector. To address this issue, a significant project funded by Masaf (Ministry of Agriculture, Food and Forestry) titled *"Protection of Biodiversity in Italian Poultry Breeds: Insights and Monitoring* (TUBAvI-2)" has been launched. The aim of this project is to recover and preserve the genetic resources of Italian poultry species *Gallus gallus* and *Meleagris gallopavo* at risk of extinction, through targeted actions to increase population size while maintaining genetic variability.

The research activities described in this doctoral thesis are part of the two aforementioned projects, which results have been published in four articles, divided into two sections.

The first section comprises two studies focused on Mediterranean trout conservation, with the goal of improving both the success of fertilization rate in artificial breeding and the understanding of the mechanisms involved in natural reproduction. This was achieved through *in vitro* studies of the effect of ovarian fluid (OF) on both cryopreserved and fresh semen.

The first study investigated the impact of OF as a natural sperm activation agent on Mediterranean trout sperm swimming performance post-thawing. The OF efficacy was compared with an activating solution (D-532) and a mixture of them (50% D-532, 50% OF). The results demonstrated that the presence of OF, either alone or combined with D-532, significantly enhanced fertilization rates with frozen sperm. Notably, 100% OF was found to be a particularly effective medium, simplifying and expediting operations in the field along riverbanks.

The second study aimed to assess the impact of native trout OF versus non-native trout OF (*S. trutta*) on the swimming performance of native males in Molise rivers. OF is crucial for fertilization success in externally fertilized fish, with interactions being species- and/or population-specific. These interactions can mitigate genetic introgression from non-native individuals. The study compared OF from native *S. cettii* females to zootechnic *S. trutta* females, evaluating their effects on sperm motility in males from the Biferno River. Our results indicate that ovarian fluids from native females (diluted at 20%) significantly improve the performance of native male sperm, unlike those from zootechnic *S. trutta*. These preliminary results suggest that OF/spermatozoa interactions could potentially influence or regulate hybridization mechanisms involving the native Mediterranean trout population in the Biferno River and the introduced domestic line of brown trout.

Session 2 includes two studies aimed at safeguarding the biodiversity of chicken and turkey breeds. The first study aims to highlight the importance of cryobanks for the semen of native breeds and their crucial role in connecting *in situ* and *ex situ* techniques to improve the effectiveness of conservation programs. In our work, activities aimed at implementing the national cryobank for chicken and turkey breeds are described, with particular attention to the identification of specific reference freezing

protocols for the respective species. The creation of the first Italian semen cryobank for native chicken and turkey breeds represents a valuable tool for the protection and conservation of biodiversity within Italian poultry populations still present in agricultural systems. The use of NMR technique in our second study has proven to be a valuable tool to deepen the understanding of the biological bases of turkey semen cryopreservation and to identify the metabolites involved in the freezing process of turkey donor sperm. This suggested the potential integration of specific metabolites in both the diet and freezing medium to enhance sperm resilience during the cryopreservation process. This discovery represents a significant contribution to the scientific community, considering the crucial importance of semen cryopreservation in the conservation and safeguarding of genetic resources. This is particularly relevant in the face of the growing threat of population decline in some breeds/species and the urgent need to counteract the risks of inbreeding to improve animal biodiversity.

RIASSUNTO

In Italia, nel corso degli anni, si è verificata una progressiva diminuzione della biodiversità animale, principalmente a causa dell'effetto combinato delle azioni dannose dell'uomo, della continua perdita e frammentazione degli habitat, dell'inquinamento e dei cambiamenti climatici. A questo riguardo, la conservazione della biodiversità animale è diventata sempre più importante nel corso degli anni, non solo dal punto di vista dell'allevamento del bestiame, che trae beneficio dalla conservazione di diverse specie, ma anche per gli animali che vivono nell'ambiente selvatico. La loro sopravvivenza è cruciale per proteggere e recuperare il prezioso patrimonio naturale sia a livello nazionale che internazionale, attraverso il mantenimento di un'elevata variabilità genetica in grado di garantire una maggiore stabilità ecologica, consentendo alle popolazioni di adattarsi ai cambiamenti climatici e ambientali.
Secondo l'Organizzazione delle Nazioni Unite per l'alimentazione e l'agricoltura (FAO), la conservazione della biodiversità può essere raggiunta attraverso l'uso di varie tecniche: *in situ* ed *ex situ*. La tecnica *ex situ* può essere ulteriormente divisa in *in vivo* e *in vitro*. La tecnica *in situ* prevede il mantenimento degli animali vivi all'interno del loro habitat naturale, mentre nella tecnica *ex situ in vivo*, il mantenimento di animali vivi avviene al di fuori del loro habitat originale. La tecnica *ex situ in vitro* consiste nella criopreservazione di materiale genetico in forma aploide (sperma e ovociti), forma diploide (embrioni, cellule somatiche) o sequenze di DNA (FAO 2012, 2015). Negli ultimi due decenni, a livello europeo, la salvaguardia delle risorse genetiche animali hanno assunto un ruolo centrale in importanti programmi di conservazione che prevedevano l'adozione di strategie sia *in situ* che *ex situ*. Tra questi, di particolare interesse è il nostro progetto europeo LIFE dal titolo "*Recupero di S. Macrostigma: Applicazione di Tecniche Innovative e Strumenti di Governance Partecipativa nei fiumi del Molise*" (Acronimo Nat.Sal.Mo). *Salmo Macrostigma* syn. *Salmo cettii* è una specie endemica dell'area mediterranea, attualmente classificata come "vulnerabile" in Europa e "in pericolo" in Italia. Le principali minacce per la specie includono l'introgressione genetica attraverso l'incrocio con ceppi zootecnici, la perdita di habitat naturali e la pesca non regolamentata. L'obiettivo principale del progetto è garantire il recupero e la conservazione della trota mediterranea nativa nei bacini dei fiumi Biferno e Volturno (regione Molise), utilizzando due tecniche innovative: la crioconservazione del seme con relativa creazione della prima criobanca europea e la tecnica dei nidi semi-naturali. Nel progetto Nat.Sal.Mo, l'uso di dosi di seme congelato provenienti dalla criobanca, combinato con opportuni schemi di fertilizzazione, si è dimostrato essere una strategia efficace per ripristinare l'integrità genetica di questa specie, consentendo di mantenere un'elevata variabilità genetica nella prole e la fitness delle popolazioni. L'altra tecnica innovativa, ovvero la costruzione dei nidi seminaturali, ha lo scopo principale di emulare il comportamento delle femmine in natura. Questo metodo prevede la semina delle uova prossime alla schiusa evitando così, l'esposizione delle

larve e dei primi stadi di vita a un ambiente artificiale. Questa tecnica assicura un maggior successo riproduttivo e riduce al minimo la pressione selettiva non adattiva.

In Italia, anche la drastica diminuzione delle razze avicole autoctone costituisce un'emergenza, questo è dovuto principalmente all'ampio utilizzo di ibridi commerciali selezionati per alte prestazioni. Per affrontare questo problema, è stato avviato un importante progetto finanziato da Masaf (Ministero dell'Agricoltura, dell'Alimentazione e delle Foreste) intitolato "*Tutela della Biodiversità nelle Razze Avicole Italiane: Approfondimenti e Monitoraggio* (TUBAvI-2)". L'obiettivo di questo progetto è quello di recuperare e conservare le risorse genetiche delle specie avicole italiane *Gallus gallus* e *Meleagris gallopavo* a rischio di estinzione, attraverso azioni mirate per aumentare le dimensioni della popolazione mantenendo nel contempo la variabilità genetica.

Le attività di ricerca descritte in questa tesi di dottorato fanno parte dei due progetti sopra menzionati, i cui risultati sono stati pubblicati in quattro articoli, divisi in due sessioni.

La prima sezione comprende due studi incentrati sulla conservazione della trota mediterranea, con l'obiettivo di migliorare sia il tasso di successo della fertilizzazione nell'allevamento artificiale sia la comprensione dei meccanismi coinvolti nella riproduzione naturale. Ciò è stato ottenuto attraverso studi *in vitro* sull'effetto del fluido ovarico (FO) sia sul seme congelato che fresco.

Il primo studio ha analizzato l'impatto del FO come agente di attivazione naturale dello sperma sulla performance di movimento degli spermatozoi di trota mediterranea dopo lo scongelamento. L'efficacia del FO è stata confrontata con una soluzione attivante (D-532) e una loro miscela (50% D-532, 50% FO). I risultati hanno dimostrato che la presenza di FO, da sola o combinata con D-532, ha notevolmente migliorato i tassi di fertilizzazione degli spermatozoi congelati. In particolare, il fluido ovarico utilizzato tal quale è risultato essere il miglior agente attivante, semplificando e accelerando le operazioni sul campo.

Il secondo studio mirava a valutare l'effetto del FO delle trote native rispetto al FO di quelle non nativa (*S. trutta*) sulla motilità degli spermatozoi dei maschi nativi dei fiumi molisani. Il FO riveste un ruolo fondamentale per il successo della fertilizzazione nei pesci a fecondazione esterna, con interazioni che variano a seconda della specie e/o della popolazione. Queste interazioni possono contribuire a mitigare l'introgressione genetica da parte degli individui non nativi. Lo studio ha confrontato il FO proveniente dalle femmine native *S. cettii* con quello delle femmine zootecniche *S. trutta*, valutandone gli effetti sulla motilità degli spermatozoi nei maschi del fiume Biferno. I nostri risultati indicano che il fluido ovarico delle femmine native (diluito al 20%) migliorava significativamente le prestazioni degli spermatozoi dei maschi autoctoni rispetto a quelli dei maschi non nativi. Questi risultati preliminari suggeriscono che le interazioni FO/spermatozoi potrebbero

potenzialmente influenzare o regolare i meccanismi di ibridazione che coinvolgono le popolazioni autoctone di trota mediterranea del fiume Biferno e la linea domestica introdotta di trota fario.
La seconda sessione comprende due studi focalizzati sulla salvaguardia della biodiversità delle razze autoctone di polli e tacchini. Il primo studio si concentra sull'importanza delle criobanche del seme delle razze autoctone e su come esse siano fondamentali nel collegare le tecniche di conservazione *in situ* ed *ex situ* al fine di ottimizzare l'efficacia dei programmi di salvaguardia della biodiversità. Nel lavoro, vengono descritte le attività finalizzate all'implementazione della criobanca nazionale per le razze di polli e tacchini, con particolare attenzione all'identificazione dei protocolli di congelamento di riferimento specifici per le rispettive specie. La creazione della prima criobanca italiana per il seme di polli e tacchini autoctoni rappresenta uno strumento prezioso per la protezione e la conservazione della biodiversità all'interno delle popolazioni avicole italiane ancora presenti nei sistemi agricoli.
Nel secondo studio, l'utilizzo della tecnica NMR si è dimostrato un prezioso strumento per approfondire la comprensione delle basi biologiche della crioconservazione del seme di tacchino. Nel lavoro sono stati identificati i metaboliti coinvolti nel processo di congelamento del seme in questa specie, suggerendo la possibilità di integrare specifici metaboliti nella dieta o nell'extender di congelamento per migliorare la resilienza degli spermatozoi durante il processo di crioconservazione. Queste scoperte offrono un contributo significativo alla comunità scientifica, data l'importanza cruciale della crioconservazione del seme nella salvaguardia delle risorse genetiche. Ciò assume particolare rilevanza alla luce della crescente minaccia di declino delle popolazioni di razze e specie avicole, nonché dell'urgente necessità di contrastare i rischi derivanti dalla consanguineità al fine di preservare la biodiversità animale.

GENERAL INTRODUCTION

Part 1

Animal Biodiversity: Definition, Primary Threats, and Current State of Resources

1. Conservation of Biodiversity

1.1 Definition and Levels of Biodiversity

One of the most significant challenges facing contemporary society is the preservation of animal biodiversity. This is a growing concern that is receiving increasing attention and scrutiny. Biodiversity refers to the variety of living organisms on planet Earth, which is currently experiencing a significant loss of species and individuals. This loss is commonly referred to as the sixth mass extinction, and it is primarily attributed to human activities (Díaz and Malhi, 2022). The decline in biodiversity has resulted in the disappearance of around 13% of bird species and 27% of mammal species (IUCN, 2023), indicating extinction rates 1,000-10,000 times higher than natural events (Bolton et al., 2022).

Biodiversity can be assessed at various levels:

- *Genetic diversity* is a measure of the inherited variability within a population. Loss of genetic diversity can seriously threaten the survival of a species, even if it is not yet extinct. It is important to maintain genetic diversity because genes that confer specific survival traits, such as disease resistance or environmental tolerance, which may be lost due to a reduction in population size caused by habitat fragmentation or other factors. Populations need genetic diversity to adapt to environmental changes. Its loss would limit options for future genetic improvement programmes globally.
- *Species diversity* describes the number and variety of organisms inhabiting a given environment, and describes the wide range of living things belonging to different groups that can interact and reproduce with each other while living in a given geographical area. Intra-species diversity refers to the genetic variations found in populations within a species, while inter-species diversity describes the variety of species present in a particular environment. Maintaining genetic diversity and protecting species from extinction-causing events, such as isolated epidemics or predators that might attack entire populations, depends on the existence of species in geographically and ecologically distinct areas. It is important to maintain a balanced distribution of species across different regions to ensure their survival.
- *Ecosystem diversity* describes the variety of communities or habitats present. It describes the wide range of organisms from various groups that can interact and reproduce with one another while residing in a particular geographic area.

In summary, biodiversity encompasses genetic diversity within species, species diversity itself, and ecosystem diversity. All these levels are interconnected and play a fundamental role in ecological balance, ecosystem resilience, and the overall well-being of our planet (Tacconi, 2000).

The conservation of animal biodiversity is becoming increasingly important, not only for agriculture, which benefits from the preservation of various species, but also for the intrinsic value of these species and for the natural heritage at both national and international levels. In recent decades, human activity has posed a significant threat to the survival of species due to environmental destruction, leading many of them towards extinction (Diavoli, 2011).

Extinction is viewed as an inevitable and natural aspect of animal species' evolutionary processes. However, the rate of extinction has increased significantly in recent decades, largely as a result of increasing human demand for natural resources and climate change. The International Union for Conservation of Nature (IUCN) has defined a number of risk categories, ranging from extinct, extinct in the wild, critically endangered, endangered, vulnerable, conservation dependent, near threatened, least concern, data deficient to not assessed, to assess the status of species for conservation purposes and in relation to their potential extinction. This categorisation system is widely used at national and international levels to assess the status of species and set conservation priorities. The IUCN Red List currently contains approximately 44,383 animal species, of which 16,928 are considered threatened with extinction. This includes 1,226 globally threatened bird species and 190 critically endangered species (IUCN, 2023).

1.2 Causes of biodiversity loss

Protecting animal species is a matter of fundamental importance for the well-being of the ecosystem and for maintaining the biodiversity balance of our planet. Animal species are an essential part of the ecosystem and play critical roles in maintaining natural equilibrium. However, many of them are threatened byextinction due to human activities, climate change, pollution, habitat destruction, and other threats. The reasons for this current situation are numerous and linked to various factors. In particular, several main ones have been identified (Soulé, 1991) that contribute to the progressive decline of biodiversity, all directly linked to human activities:

1. *Habitat destruction:* The destruction, fragmentation, and degradation of natural habitats due to activities such as deforestation, urbanization, agriculture, and infrastructure development. This disrupts ecosystems and reduces available habitats for different species, leading to biodiversity loss.
2. *Climate change:* Alteration of global climate patterns due to human activities, such as burning fossil fuels, deforestation, and industrial processes, results in changes in temperature,

precipitation, and other environmental factors. These changes can have negative effects on species' survival, disrupt ecosystems, and contribute to biodiversity loss.

3. *Pollution:* Various forms of pollution, including air, water, and soil pollution, can have detrimental effects on biodiversity. Pollution from industrial processes, agricultural runoff, and improper waste management can poison or disrupt organisms' reproductive systems, leading to declines in populations and species diversity.
4. *Overexploitation:* The unsustainable extraction or use of natural resources, such as overfishing, illegal hunting, and deforestation, can deplete species populations and disrupt ecosystems. Removing species from ecosystems at an unsustainable rate can lead to imbalances and cascading effects on other species.
5. *Invasive species*: The introduction of non-native species into new environments can have severe impacts on native species and ecosystems. Invasive species often compete with native species for resources, prey on them, or introduce diseases, leading to declines in native populations and reduced biodiversity.
6. *Agricultural practices:* Intensive agricultural practices, such as monoculture farming, intensive pesticide use, and the loss of traditional cultivation methods, can harm biodiversity. These practices often involve the loss of natural habitats, the use of harmful chemicals for non-target organisms, and the reduction of genetic diversity in crops and livestock.
7. *Population growth and urbanization:* Rapid human population growth and urban development contribute to the conversion of natural habitats into human-dominated landscapes. This expansion leads to habitat loss, fragmentation, and increased pressure on ecosystems, resulting in biodiversity loss.
8. *Lack of awareness and conservation efforts:* The lack of awareness and understanding of the value and importance of biodiversity can hinder conservation efforts. Insufficient conservation measures, weak legislation, and ineffective enforcement of environmental regulations can exacerbate biodiversity loss.

It is important to note that these causes are often interconnected and can reinforce each other, leading to cumulative effects on biodiversity loss. Addressing these factors requires a comprehensive approach that includes sustainable land use practices, conservation strategies, education, and international cooperation. The importance of preserving biodiversity on our planet has been universally recognised. A broad consensus in both the scientific community and society has driven governments to prioritise the maintenance of biological diversity through habitat protection and species conservation programmes. Many countries have introduced new laws to curb this problem, halt the exploitation and mismanagement of animal resources. Species protection is implemented not

only through national and European community laws but also through international agreements and treaties. These agreements are necessary for several reasons: many species migrate across state borders, and there is international trade in animals and derived products, requiring international cooperation to ensure species conservation.

In conclusion, the conservation of animal biodiversity represents a crucial challenge for our society. Protecting species and their habitats requires a combination of national and international policies, laws, agreements, and concrete actions. Collaboration among governments, scientists, environmental organisations, and the public is essential to preserving biological diversity and ensuring a sustainable future for the animal species on our planet.

1.3 Conservation of Animal Species

The conservation of animal species is one of the most important challenges of our time, as our planet's biodiversity is threatened by human activities, climate change and pollution. Animal species are a fundamental pillar of terrestrial and marine ecosystems and play a key role in regulating ecological balances. Their conservation is essential for the health of the environment and the well-being of future generations. The acceleration of biodiversity loss is now widely recognised, with a significant increase in the number of species listed as critically endangered (e.g. from 168 to 232 bird species, for fish species from 157 to 834) or endangered (e.g. from 235 to 405 bird species, for fish species from 134 to 1,340) between 1996 and 2023, according to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (2023). These data highlight the global emergency of biodiversity loss and the growing threat to many animal species. The rate at which species are going extinct is alarming and requires immediate and decisive action to reverse this trend.

The conservation of animal species is crucial for several reasons. Firstly, species contribute to the biological diversity of the planet, creating an ecological fabric that allows ecosystems to function. Each species has a role in the food chain and contributes to ecological balance. The loss of one species can have effects on others, compromising the entire ecosystem. Secondly, many species have cultural and historical value, being a source of inspiration for art and spirituality. The species are threatened by various factors, as described in the previous chapter, and to preserve them, strategies are adopted such as the creation of protected areas, compliance with laws against hunting and illegal trade, constant monitoring of populations and public awareness. International cooperation is crucial to manage migration and fight illegal trafficking.

These collective actions can contribute to the conservation of species and the protection of biodiversity for future generations.

1.4 Status and trends of animal genetic resources

The status and trends of animal genetic resources are of great importance for conserving genetic diversity and the sustainability of animal species. Assessing the state of these resources provides crucial information about the genetic diversity present in animal populations and the evolution of genetic variations over time. Such information is essential for developing conservation and management strategies for animal species, as well as for adapting breeding and genetic improvement practices to preserve genetic diversity and maintain the resilience of animal populations.

Currently, the Global Databank database contains data from 182 countries (and 15 dependent territories) related to 37 species. In the considered period, the overall number of registered national breed populations in the Global Databank has slightly increased, going from 15,115 in 2021 to 15,313 in 2022 (Table 1). As of September 2022, the total number of registered national populations of mammals was 11,555, compared to 11,409 in February 2021. As for birds, in 2022, there were 3,758 registered national populations, compared to 3,706 in 2021 (FAO 2023).

Table 1- Status of information recorded in the global databank for animal genetic resources. *(FAO 2023)*

Year of analysis	Mammalian species		Avian species		Countries covered
	Number of national breed populations	Proportion with population data (%)	Number of national breed populations	Proportion with population data (%)	
1993	2719	53	–	–	131
1995	3019	73	863	85	172
1999	5330	63	1049	77	172
2006	10512	43	3505	39	181
2008	10550	52	3450	47	181
2010	10507	54	3414	47	182
2012	10712	57	3482	48	182
2014	11062	60	3807	56	182
2016	11116	61	3799	57	182
2018	11371	62	3689	58	182
2021	11409	66	3706	61	182
2022	**11555**	**66**	**3758**	**63**	**182**

No data recorded for Andorra, Brunei Darussalam, Holy See, Liechtenstein, Marshall Islands, Micronesia (Federated States of), Monaco, Nauru, Qatar, San Marino, Singapore, South Sudan, Timor-Leste, United Arab Emirates, Western Sahara.

Since 2021, the percentage of national populations of avian species for which some population data are available has increased from 61% to 63%, while for mammals, the proportion has remained stable around 66%.

Figure 1 presents the proportions and numbers of national breed populations for which population data have been reported at least once in the past, divided by region. The level of activity in data updating varies considerably between countries and regions (Figure 2). As shown in Figure 2, 63 countries and territories have updated census size information for at least one of their national breed populations since the beginning of 2019. For 17 countries, the last update was made between 2015 and 2018, and for seven countries, the last update was between 2013 and 2014. For these seven

countries, this means that if no updates are provided in the next two years, all breeds will be classified with an "unknown" risk status when calculating official indicators for the risk of extinction and in the upcoming report on status and trends. For 110 countries and territories, there have been no population updates since 2012. (FAO 2022).

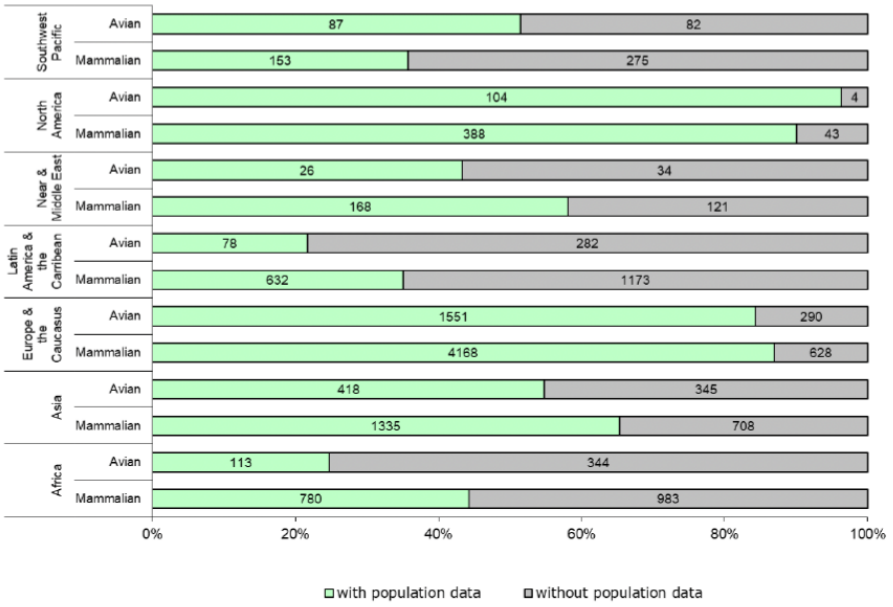
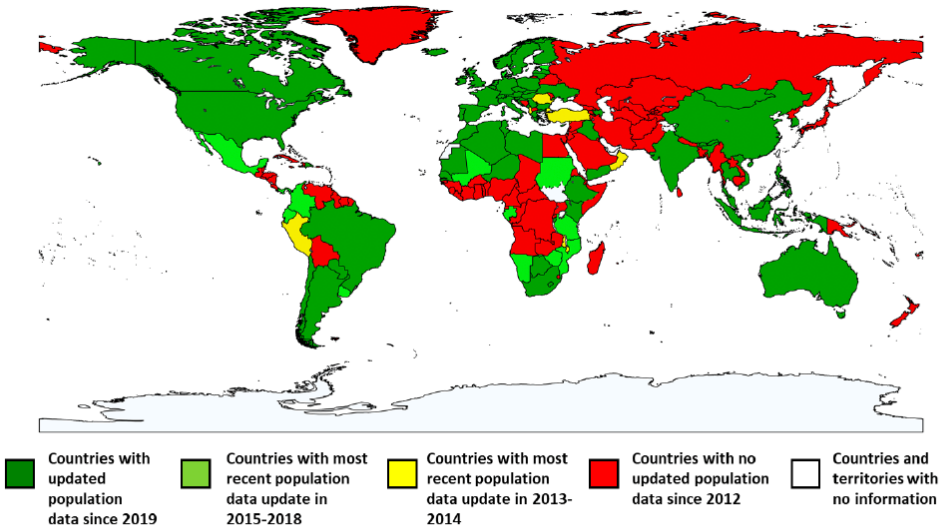


Figure 1- Proportions (% - relative length of colored bars) and numbers of national breed populations for which population data have been reported per region. *(FAO 2023)*



Source: United Nations Geospatial. 2020. Map of the World. United Nations. Cited 22 August 2022. www.un.org/geospatial/file/3420/download?token=TUP4yDmF modified with DAD-IS; https://www.fao.org/dad-is

Notes: Final boundary between the Sudan and South Sudan has not yet been determined. Dotted line represents approximately the Line of Control in Jammu and Kashmir agreed upon by India and Pakistan. The final status of Jammu and Kashmir has not yet been agreed upon by the parties.

Figure 2- The timing of the most recent population updates for each country. *(FAO 2023)*

1.5 Biodiversity in the livestock sector

In the context of animal husbandry, genetic diversity is the main concept of biodiversity. Only about 40 of the over 5,000 species of mammals and birds currently known to humans have been domesticated or otherwise developed into livestock, making up the remaining Animal Genetic Resources (AnGR) (FAO, 2000). The results of natural selection in combination with thousands of years of selective breeding have produced significant genetic diversity among livestock populations all over the world. Multipurpose breeds raised by small-scale farmers and herders, primarily in low-input production systems, coexist with high-performance animals that are intensively bred to produce more and more uniform products under controlled management conditions. These components make up "Domestic Animal Diversity" (DAD), or DAD for short.

According to the FAO, it is crucial to manage this animal genetic diversity effectively in order to ensure global food security, support wholesome diets, improve rural livelihoods, increase community and individual resilience, encourage sustainable development, and protect the livelihoods of hundreds of millions of people. The rapid rise in demand for products made from animals in many developing nations over the past 20 years, however, has resulted in general economic development, which has decreased the genetic diversity of animals. A joint report by the FAO, the International Food Policy Research Institute (IFPRI), and the International Livestock Research Institute (ILRI) predicted this inevitable process of genetic erosion, driven by the rising consumer demand for products derived from animals, as early as 1999 and dubbed it the "Livestock Revolution" (Delgado, 2003).

According to the analysis of the most recent global data, the status of Animal Genetic Resources for Food and Agriculture in the World (FAO, 2022) reported that out of 8,803 different livestock breeds worldwide, 2,133 breeds are categorised as being at risk (24% of all breeds, including extinct ones); meanwhile, about 59% of the breeds are categorised as having an unknown risk status (Figure 4). Numerous factors could endanger genetic diversity in developing nations, and many authors appear to agree on the broad trends and dangers to AnGR. The use of exotic germplasm, changes in production systems, changes in producers' preferences as a result of socioeconomic factors, various disasters (droughts, famines, disease epidemics, civil conflicts/war), specialisation (emphasis on a single productive trait), genetic introgression, technological and biotechnological development, and political instability are the primary causes of genetic erosion. (Rege and Gibson, 2003; Tisdell, 2003; Iñiguez, 2005; Floros et al., 2010).

The FAO claims that the biggest threat to traditional livestock production systems and the local breeds they are associated with is probably their marginalisation. This threat is primarily brought on by the rapid spread of large-scale intensive livestock production and the use of a limited number of breeds. Increasingly, the world's production of meat, milk, and eggs is dependent on a small group of high-

yielding breeds, which are mainly employed in industrial production systems. The ease with which genetic material, production technologies, and inputs can now be transported around the world has clearly aided these processes of intensification and industrialization (FAO, 2007).

In this scenario, the wise management of global livestock biodiversity is becoming an increasingly significant challenge for the international community.

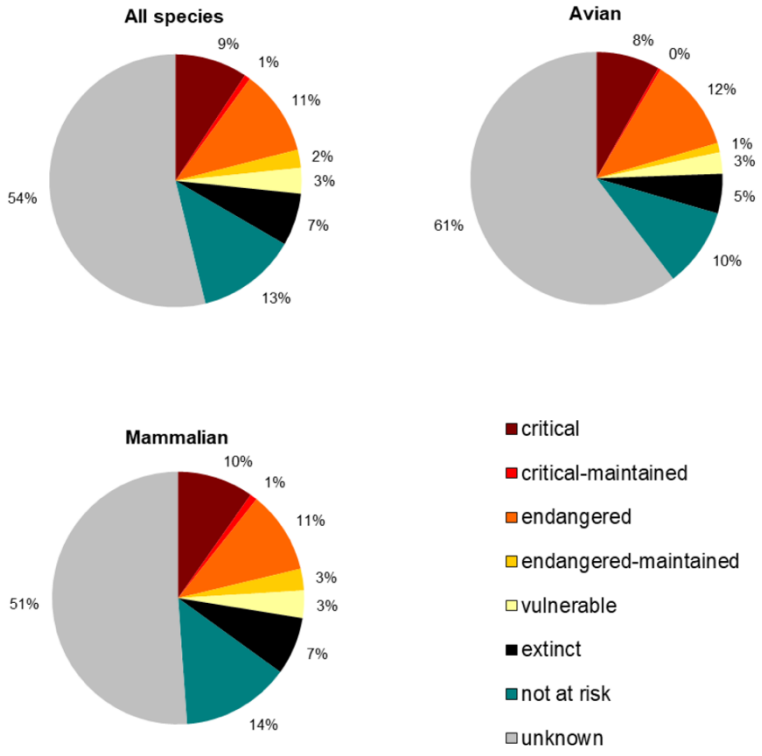


Figure 3 - Proportions of the world's breeds by risk status category. *(FAO, 2019)*

1.5.1 The relevance of local native breeds

Indigenous breeds refer to populations of domestic animals that have adapted and naturally evolved in a specific geographical area over a long period, often spanning centuries or even millennia.

Local breeds represent both a cultural and biological heritage, the result of years of agricultural tradition that reflects the history of rural populations' cultures. Additionally, they are invaluable resources for scientific research in the field of genetics (FAO 2012).

The diversity of animal breeds is a fundamental asset for the planet's future because each breed is an expression of a specific environmental, historical, and cultural context in which they originated. Unfortunately, contemporary industrial farming relies on a very limited number of selectively bred breeds that are highly productive and suitable for intensive farming. This puts indigenous breeds at risk of disappearing rapidly and in an entirely unnatural manner.

Currently, the survival of local breeds is linked to various factors, including their hardiness, which means they are better adapted to challenging environmental conditions, their higher market value compared to industrial-type productions, and the superior quality of their products (Papachristoforou et al., 2013). The conservation of local breeds is closely tied to the concept of sustainable development, where the protection of genetic resources goes hand in hand with increasing the income of livestock farmers. Based on this, the valorization of biodiversity could lead to the development of initiatives aimed at producing and marketing high-quality or "typical products" in parallel with the conservation efforts and securing various endangered breeds. In this way, the connection to the territory and the culture from which a particular product originates would also be valued.

Traditionally, local farmers have been the custodians of this animal genetic diversity. Despite the intensification of agriculture and international trade in livestock, local breeds are still considered the basis for selection for efficient food production in changing environments (FAO, 2007, 2010 b).

Thanks to their unique characteristics, such as tolerance/resistance to various diseases (including severe enteric and ectoparasitic parasites), tolerance to extreme climatic factors, or the provision of specialised products (Hammond and Galal, 2000), these breeds can be particularly suitable for coping with the future emergence of new and virulent animal diseases, rapid climate changes, selection errors, unforeseen catastrophic events, and changes in consumer preferences.

The primary critical point of local breeds generally lies in their low productivity. This means that a significant part of the animals' body resources is allocated to maintaining vital functions, such as kinetic activity, foraging behavior, and immune response, with only a residual amount dedicated to production traits. Although local breeds may not be competitive in terms of production, they can find effective application in extensive livestock systems (Castellini, 2005; Castellini et al., 2008), where greater kinetic activity and adaptation to less protected environments are required (Dal Bosco et al., 2021). The sustainable use of local breeds in production systems offers important ecosystem services. Among these are provisioning services, such as the supply of food and genes, but also cultural services, such as the preservation of cultural heritage and identity, and regulating services, such as landscape and biodiversity management. These services constitute essential sources of income to revitalize rural areas (Ovaska and Soini, 2017). In rural areas, new forms of rural entrepreneurship are emerging and expanding, harnessing local breeds, such as tourism and Green Care (Hassink et al., 2005; MA, 2005; Ovaska and Soini, 2017). As a result, the ongoing erosion of these valuable animal genetic resources would lead to a reduction in development opportunities for rural economies in some countries, as well as negative social and cultural impacts due to the longstanding connection between local breeds and the culture of rural communities. The replacement of indigenous breeds could result in the loss of products and services cherished by the local population, necessitating a broader

consideration of the conservation of local breeds to support rural communities and their economic foundations. Furthermore, such losses could limit future development options based on products and services derived from specific breeds, which could otherwise add significant economic value as consumer demands become increasingly diversified (FAO, 2022).

The diversity of animal breeds is a fundamental asset for the future of the planet because each breed is the expression of a specific environmental, historical, and cultural context in which they originated. Today, unfortunately, industrial farming relies on a very limited number of selectively bred breeds that are highly productive and suitable for intensive farming. This puts indigenous breeds at risk of disappearing rapidly and entirely unnaturally.

The protection and promotion of indigenous breeds are crucial for preserving biological diversity, supporting sustainable agriculture, and keeping local traditions alive. International organizations like the FAO (Food and Agriculture Organization of the United Nations) advocate for the conservation and sustainable use of indigenous breeds through genetic conservation and rural development programs (Gandini and Oldenbroek, 2007).

2. Fish Biodiversity

The diversity of fish is significant for various reasons. It encompasses a broad range of species adapted to varying environmental conditions. Fish are valuable indicators of trends in aquatic diversity, reflecting the conditions of their habitats. Additionally, they significantly influence the distribution and abundance of other species within aquatic ecosystems. The diversity of species among fish is an integral part of the global natural heritage and provides essential ecosystem services to millions of people, especially in the context of commercial and recreational fishing. Interestingly, despite the majority of the Earth's surface being covered by water and oceans having a significant average depth, the greatest diversity of fish species is found in freshwater environments, such as continental shelves, coral reefs associated with islands, and freshwater bodies like rivers and lakes (Moyle and Leidy, 1992). It is estimated that the total number of all fish species is 32,500 (Nelson, 2006), with over 15,000 species of freshwater fish, a surprisingly high number considering that freshwater constitutes less than 0.3% of the available global water. While marine communities contain a greater total number of species, freshwater environments are much richer per unit volume of habitat, thanks to their productivity, physiographic diversity, and geographical isolation (Ormerod, 2003). Freshwater fish represent approximately 25% of all vertebrates, making a significant contribution to global biodiversity (Reid et al., 2019). However, according to the latest edition of the Red List of the International Union for Nature Conservation (IUCN) published in 2023 on freshwater fish species are globally in serious danger. The primary threats to global freshwater fish biodiversity primarily stem from human activity and can be grouped into five interacting categories: overexploitation, water pollution, flow modification, habitat destruction or degradation, and invasion by alien species. These factors lead to the decline of native populations and a reduction in their distribution area (Allan and Flecker, 1993; Naiman and Turner, 2000; Jackson et al., 2001; Malmqvist and Rundle, 2002; Rahel, 2002; Revenga et al., 2005; Dudgeon et al., 2006).

The protection of aquatic biodiversity presents a significant challenge for conservation, especially considering the ongoing growth of human populations. The sustainable use of fishery resources has become essential to preserving fish diversity and the health of aquatic ecosystems. While over two-thirds of the Earth's surface is covered by water and oceans have a vast expanse, it is noteworthy that both surface and deep ocean waters, despite their vastness, are relatively uniform habitats with low productivity and a limited number of species. In contrast, the greatest diversity of aquatic life is predominantly distributed along continental shelves, in coral reefs associated with islands, and in freshwater environments (Moyle and Leidy, 1992).

2.1 The state of the fishery resources: in particular the Mediterranean brown trout

Freshwater ecosystems, among all the natural environments on our planet, are among the most vulnerable and threatened. Over the past few decades, we have witnessed a dramatic decline in their extent and a decrease in biodiversity in these precious regions. This chapter will explore the critical situation of freshwater ecosystems worldwide, highlighting the importance of protecting and preserving them for future generations. The decline of freshwater ecosystems has been staggering. From 1997 to 2011, the area of these habitats decreased by 64% globally, while in Europe, from 1970 to 2008, we saw a 50% decrease (Costanza et al., 2014; IPBES, 2018; Gozlan et al., 2019). These data are alarming and underscore the urgency of taking action to protect these vital environments.

Freshwater ecosystems are biodiversity oases. Over 10,000 species of fish, representing 40% of global fish diversity and a quarter of global vertebrate diversity, call these waters home (Lundberg et al., 2000). When including amphibians, aquatic reptiles like crocodiles and turtles, and mammals like otters, river dolphins, and platypuses, we discover that up to a third of all vertebrate species depend on freshwater habitats. In the 1980s, the burgeoning field of conservation biology led to a greater emphasis on safeguarding freshwater ecosystems and organisms (Soule et al.,1995). Researchers conducted extensive studies to gain a clearer understanding of the effects of overexploitation and identify ways to mitigate them. However, despite the abundance of research, numerous uncertainties persist. To address threats to freshwater ecosystems, significant efforts have been made to assess the status of freshwater species at international and national levels. Such assessments have provided important information but often focus on specific taxa or fail to record population collapses. These limitations can lead to the invisible collapse of other freshwater species.

Europe is particularly rich in freshwater ecosystems. The region hosts 546 native species of freshwater fish (Kottelat and Freyhof, 2007). Only in the last 20 years, the European Mediterranean region has been recognized as a hotspot of this diversity. Freshwater fish in Europe encompass a variety of taxonomic and ecological groups, including Cypriniformes and Salmoniformes, which dominate warm waters and mountainous regions, respectively (Table 2). Cypriniformes adapted to warm waters, such as carp, chub, dace, and loaches, exhibit the highest level of species diversity in the southern and temperate parts of Europe, while Salmoniformes, like salmon, trout, grayling, and whitefish, are more abundant and diversified in mountainous regions and northern Europe. Both groups are known to include many locally endemic species at the species level.

Table 2 - Diversity and endemism in freshwater fish families. *(Freyhof and Brooks, 2011)*

Class	Order	Family	Europe			EU 27		
			Number of species	Number of Endemics	% Endemics	Number of species	Number of Endemics	% Endemics
Actinopterygii	Acipenseriformes	Acipenseridae	8	2	25%	7	1	14%
	Anguilliformes	Anguillidae	1	0	0%	1	0	0%
	Atheriniformes	Atherinidae	1	0	0%	1	0	0%
	Clupeiformes	Clupeidae	18	10	56%	11	4	36%
	Cypriniformes	Balitoridae	8	7	88%	5	3	60%
		Cobitidae	35	31	89%	25	19	76%
		Cyprinidae	237	205	86%	162	127	78%
	Cyprinodontiformes	Cyprinodontidae	4	3	75%	4	3	75%
		Valenciidae	2	2	100%	2	2	100%
	Esociformes	Esocidae	1	0	0%	1	0	0%
		Umbridae	1	1	100%	1	0	0%
	Gadiformes	Lotidae	1	0	0%	1	0	0%
	Gasterosteiformes	Gasterosteidae	8	4	50%	7	3	43%
	Mugiliformes	Mugilidae	5	0	0%	5	0	0%
	Osmeriformes	Osmeridae	3	1	33%	1	1	100%
	Perciformes	Blenniidae	2	1	50%	2	1	50%
		Gobiidae	43	29	67%	22	8	36%
		Moronidae	1	0	0%	1	0	0%
		Percidae	15	12	80%	13	5	38%
	Pleuronectiformes	Pleuronectidae	3	1	33%	2	0	0%
	Salmoniformes	Salmonidae	102	93	91%	80	71	89%
	Scorpaeniformes	Cottidae	16	14	88%	16	11	69%
	Siluriformes	Siluridae	2	1	50%	2	1	50%
	Syngnathiformes	Syngnathidae	1	0	0%	1	0	0%
Cephalaspidomorphi	Petromyzontiformes	Petromyzontidae	13	9	69%	8	3	38%
Total			**531**	**426**	**80%**	**381**	**263**	**69%**

* This table includes all species that were assessed as part of this project, and that are native or were naturalised before AD 1500; species introduced after this date are not included. Species of marginal occurrence in Europe are included. For the EU 27 assessment those species which do not occur in the EU are excluded.

Two regional levels were used to assess the IUCN Red List status of freshwater fish in Europe: geographical Europe and the EU's 27 current member states (EU 27).

This assessment finds that at least 37% of species are threatened at the European level (39% in the EU 27), 12% are critically endangered (13% in the EU 27), 10% are endangered (11% in the EU 27), 15% are vulnerable at both the regional and global levels, and 4% are near threatened (Figure 4 A-B). Consequently, more than a third of the freshwater fish species in Europe face a threat. This indicates the enormous problem with conservation as it represents the second highest threat level of any taxonomic group evaluated thus far.

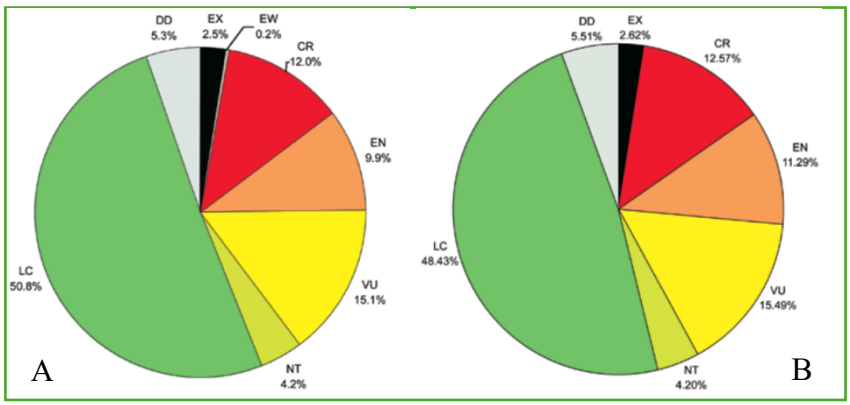


Figure 4- IUCN Red List status of freshwater fishes in (A) Europe and in (B) EU27. *(Freyhof and Brooks, 2011)*

According to Table 2 the Red List status is broken down by taxonomic family, with each family having a different number of species and threat level for those species. The Acipenseridae, Anguillidae, Cypronodontidae, and Umbridae families are the most endangered, followed by Blennidae, Cobitidae, Cyprinidae, and Salmonidae. 41% of the species in the family Salmonidae are endangered. According to Table 3, out of the 98 European species, 8 are thought to be extinct, 1 is extinct in the wild, 9 are critically endangered, 7 are endangered, 24 are vulnerable, 2 are near threatened, 32 are of least concern, and 15 are data deficient.

Table 3 - IUCN red list status (at the european level) of freshwater fishes by taxonomic family, including salmonidae family (in green box) *(Freyhof and Brooks, 2011)*

Family	Total*	EX	EW	CR	EN	VU	NT	LC	DD	% Threatened*
ACIPENSERIDAE	8			7		1				100%
ANGUILLIDAE	1			1						100%
ATHERINIDAE	1							1		0%
BALITORIDAE	8			1		1		6		25%
BLENNIIDAE	2			1				1		50%
CLUPEIDAE	18			3	1	3		10	1	39%
COBITIDAE	35			5	5	6	3	16		46%
COTTIDAE	16			1		2		10	3	19%
CYPRINIDAE	236	3		23	35	38	14	119	4	41%
CYPRINODONTIDAE	6			3	2			1		83%
ESOCIDAE	1							1		0%
GASTEROSTEIDAE	8	1		1				6		13%
GOBIIDAE	43			4	2	3	1	30	3	21%
LOTIDAE	1							1		0%
MORONIDAE	1							1		0%
MUGILIDAE	5							5		0%
OSMERIDAE	2							2		0%
PERCIDAE	15			3			1	10	1	20%
PETROMYZONTIDAE	13	1		1			1	10		8%
PLEURONECTIDAE	3							3		0%
SALMONIDAE	98	8	1	9	7	24	2	32	15	41%
SILURIDAE	2							1	1	0%
SYNGNATHIDAE	1							1		0%
UMBRIDAE	1					1				100%
Total	**525**	**13**	**1**	**63**	**52**	**79**	**22**	**267**	**28**	**37%**

IUCN Red List Status: EX – Extinct, EW – Extinct in the Wild, CR – Critically Endangered, EN – Endangered, VU – Vulnerable, NT – Near Threatened, LC – Least Concern, DD – Data Deficient.
* Does not include species classed as Not Applicable (NA)

The brown trout is part of a complex of incipient species, known as the *Salmo trutta* species complex, originally inhabiting freshwater environments in the Palearctic region (Talarico et al., 2023). Currently, the brown trout is one of the most endangered freshwater fish species in the Mediterranean area. This group of species has a wide native distribution in Eurasia and North Africa (Tougard, et al. 2018) and includes the surviving native populations on the Tyrrhenian and Adriatic sides of the Italian peninsula (Lorenzoni et al., 2019). Currently, four closely related species of trout are recognized in the Italian peninsula and Mediterranean islands: *Salmo fibreni*, *S. carpio*, *S. cettii*, and *S. marmoratus*,

in addition to a taxonomically complex and understudied group of brown trout relatives in Morocco and Algeria (Lobón-Cerviá et al., 2019).

The four Italian trout species exhibit significant morphological and life history differences. For the most part, they are distributed allopatrically, but they may overlap in some watercourses. S. *marmoratus* and *S. cettii* are found in streams with broad distributions. *Salmo marmoratus* inhabits the tributaries of the Po River flowing from the European Alps. *S. cettii* is found in Mediterranean rivers of the Apennines, Sardinia, Sicily (Splendiani et al. 2016), and Molise (Iaffaldano et al., 2016 a). There are several isolated populations in the western Alps, but their presence in the Po River system is a subject of controversy. *S. fibreni* and *S. carpio* are original endemic species dwelling in lakes. *S. fibreni* occurs in the small Posta Fibreni Lake in central Italy, while *S. carpio* inhabits the relatively large and warm Lake Garda in the northern Po Valley (Lobón-Cerviá et al., 2019).

All native trout species in Italy are included in national or international red lists, with slight differences related to different geographical regions, evaluation periods, or basic criteria (Table 4). *S. carpio* and *S. fibreni* are considered to be of critical status due to illegal fishing, the introduction of non-native species (such as *Coregonus sp*. in Lake Garda), pollution, and habitat degradation. For *S. cettii* and *S. marmoratus*, the primary threat is hybridization with non-native brown trout (Schöffmann et al., 2007; Fruciano et al., 2014; Splendiani et al., 2016). Non-hybridized *S. cettii* are absent from the central and eastern Alps but are distributed as 20.0% in the western Alps, 2.8% in the Apennines, 50.0% in Sardinia, and 33.3% in Sicily (Splendiani et al., 2016). Streams with non-native invasive brown trout or hybridized native trout populations are common in the eastern (94.3%), central (100%), and western Alps (60.0%) and less common in the Apennines (59.2%) and Sardinia (50.0%), but Sicily remains relatively unaffected by non-native genetic lineages. For *S. marmoratus*, all populations in the central Alps are hybridized, but original populations persist in the western (75%) and eastern (100%) Alps (Splendiani et al., 2016).

Table 4 - Conservation status of Italian native trout

Species	IUCN Red List of Threatened Species	Italian Red List of Fish	Italian Red List of Vertebrates
Salmo carpio	CR	CR	EN
S. cettii	NT	CR	CR
S.fibreni	VU	CR	CR
S.marmoratus	LC	EN	CR

ABBREVIATIONS: CR = CRITICALLY ENDANGERED, EN = ENDANGERED, VU = VULNERABLE, NT = NEAR THREATENED, AND LC = OF LEAST CONCERN *(Lobón-Cerviá et al., 2019)*

3. Poultry Biodiversity: Importance and main threats

Today, poultry farming represents one of the most dynamic livestock production sectors, both globally and in our country. Market preference for poultry meat is driven by numerous advantages over other sectors. Among the various characteristics, we can first emphasize accessibility since the cost per kilogram of poultry meat is significantly lower than that of pork, beef, or lamb meat. Furthermore, poultry farming offers greater convenience, is not subject to religious restrictions, and is associated with a healthy eating image due to its low-fat content and balanced protein intake (Mottet et al., 2017). This perspective is confirmed by data provided by the OECD-FAO, which indicate that global meat production contracts annually for nearly all traditionally raised species, except for poultry, which continues to grow steadily, even during the recent COVID-19 pandemic (Hafez, H.M. et al., 2020). In intensive production contexts, the most common poultry species is domestic chicken (*Gallus gallus*) followed by turkeys (*Meleagris gallopavo*), while other species such as ducks, geese, quails, pigeons, etc., are present in much smaller quantities. Among these species, chickens and turkeys are the primary sources of poultry meat and eggs.

Poultry farming also stands out for its reduced environmental impact compared to other animal production sectors. This is attributed to its high efficiency in converting feed into eggs and meat. The short production cycle and targeted genetic selection to improve performance provide a significant advantage over the raising of ruminants or swine. This efficiency is particularly evident in the poultry meat industry, where modern broiler chickens reach slaughter weight in cycles of only about 40 days, yielding a high meat output (Bohrer B.M. 2017).

Turning to the data on animal breeds, a total of 8.803 breeds have been recorded based on the most recent global data (FAO, 2022), of which 7.745 are local breeds and 1.058 are transboundary breeds. Among the transboundary breeds, there are 511 regional transboundary breeds (registered in a single region) and 547 international transboundary breeds (reported in more than one region).

Regarding poultry breeds, Europe and the Caucasus host the highest number of regional transboundary breeds, with a total of 68 different varieties, compared to fewer than 10 in each of the other regions. In every region of the world, the numbers of registered local breeds for poultry species are reported (Table 5). Specifically, Europe and the Caucasus are the areas with the highest number of indigenous chicken and turkey breeds, representing a significant percentage of the global total for these breeds.

It's important to note that a total of 2.199 breeds are considered at risk, which corresponds to 24% of the total breeds, including extinct ones, according to the Domestic Animal Diversity Information System (DAD-IS) by FAO. However, approximately 59% of breeds are categorized as having

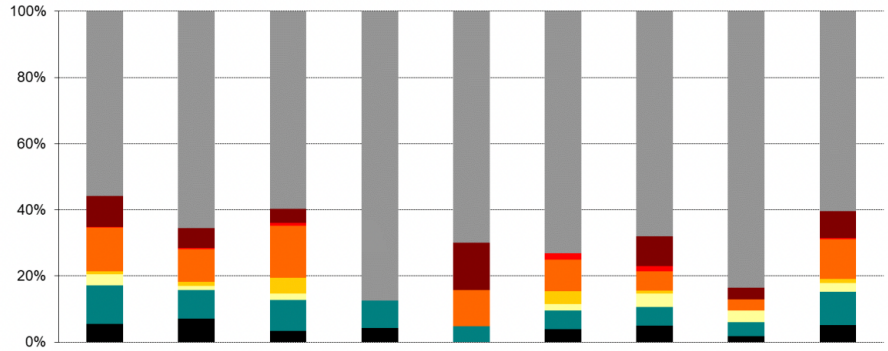
uncertain risk status. The lack of data on the risk status for some breeds (13 in total) represents a significant limitation in the planning and implementation of breed conservation activities.

Table 5 - Number of reported local avian breeds in the world including chicken (in red box) and turkey (in blue box) *(FAO, 2023)*

Species	Africa	Asia	Europe & the Caucasus	Latin America & the Caribbean	Near & Middle East	North America	Southwest Pacific	World
Chicken	138	326	802	62	33	11	37	1409
Duck	16	101	111	20	3	1	12	264
Goose	10	44	118	5	2		2	181
Muscovy duck	4	8	6	1	1		2	22
Pigeon	7	13	42	7	8	1	2	80
Quail	3	23	19	4				49
Turkey	11	11	46	9	2	8	5	92
Others	44	24	24	7	2		1	102
Total	**233**	**550**	**1168**	**115**	**51**	**21**	**61**	**2199**

Note: Figures exclude extinct breeds. Figures for cassowary, Chilean tinamou, duck × Muscovy duck, emu, guinea fowl, ñandu, ostrich, partridge, peacock, pheasant and swallow are combined in the "others" category.

Among poultry species, chickens have by far the highest number of breeds at risk on a global scale (Figure 5). It has been reported that out of 1,666 local chicken breeds, 931 are classified as unknown, 156 as critical, 221 as endangered, and 91 as extinct. As for turkeys, 11 breeds are considered critical, 7 are classified as endangered, 2 breeds are extinct, while 83 local turkey breeds are categorised as "unknown".



	Chicken	Duck	Goose	Muscovy duck	Pigeon	Quail	Turkey	Other	Total
Unknown	931	205	126	21	58	38	83	97	1562
Critical	156	19	9	0	12	0	11	4	211
Critical-maintained	3	1	2	0	0	1	2	0	9
Endangered	221	31	33	0	9	5	7	4	310
Endangered-maintained	14	4	10	0	0	2	1	0	31
Vulnerable	56	4	4	0	0	1	5	4	71
Not at risk	194	27	20	2	4	3	7	5	262
Extinct	91	22	7	1	0	2	6	2	131
Total	**1666**	**313**	**211**	**24**	**83**	**52**	**122**	**116**	**2587**

* Other: duck × Muscovy duck crossings, Chilean tinamou, cassowaries, emus, ñandus, peacocks and swallows.

Figure 5 -Risk status of the world's avian breeds in September 2022: absolute (table) and percentage (chart) figures by species (species refer to both the bar above and numbers below. *(FAO 2023)*

3.1 Poultry Genetic Resource in Italy

The recovery of Italian native poultry breeds plays a crucial role in the context of agriculture and biodiversity conservation in Italy. The growing demand for poultry products has led to a significant increase in production, but this trend has also had a negative impact on the conservation of native poultry breeds in Italy. The preference for high-yield commercial hybrids has caused a drastic reduction in the breeding of local breeds.

Italian native poultry breeds represent a valuable agricultural and cultural heritage characterized by unique genetic traits. These traits have been developed through processes of natural selection and coevolution that have extended over centuries and continue to this day. These breeds stand out for their disease resistance and their ability to survive under limited or low-quality feeding conditions. Their preservation significantly contributes to the conservation of genetic diversity within poultry species. Furthermore, the conservation of local breeds is of paramount importance to meet unforeseen future needs in terms of poultry breeding and production, as emphasized by Chen et al. in 2019, and Zhang et al. in 2020. This conservation not only concerns genetic value but also has a significant socioeconomic, cultural, and ecological impact.

However, the pressure from globalized economies and the demand for high yields have put the survival of many of these breeds at risk. Breeding native breeds is often characterized by limited production, making it challenging to compete with commercial hybrids in terms of quantity produced. Furthermore, food hygiene standards and product uniformity requirements have limited opportunities for small-scale poultry breeders to market their products (Rischkowsky and Pilling, 2007).

Thoughts on cultural, social, and environmental values are increasingly being considered alongside economic factors by farmers, breeders, and consumers as trends shift. This has sparked a resurgence in interest in protecting indigenous poultry breeds.

In Italy, the issue of biodiversity loss in the poultry sector has been addressed through agricultural policies aimed at the conservation of livestock species. Over the last 20 years, plans for the preservation of local poultry breeds have been implemented, initially at the regional level and subsequently extended to national policies, as described by Cendron et al. in 2020. These efforts are crucial for preserving the genetic diversity of Italian poultry breeds and ensuring their long-term survival.

Currently in Italy, according to the Food and Agriculture Organization (FAO), 53% of native livestock breeds in Europe and the Caucasus are at risk of extinction. Specifically, concerning local chicken breeds in Italy, out of 53 recognized breeds, 67% are already extinct, while 21% are at risk of extinction. (FAO 2022) In Italy, out of 53 recognized local chicken breeds, 67% are already extinct, while 21% are at risk of extinction. (Castillo et al. 2021).

The FAO has classified 18 Italian chicken breeds as endangered or critically endangered (FAO 2020). In recent years, a National Registry has been established. The Genealogical Book of native poultry breeds has been entrusted to ANCI (the Italian National Association of Breeders) by the Ministry of Agricultural, Food, and Forestry Policies with Ministerial Decree No. 38992 dated December 5, 2019. The Book represents the natural continuation of the Register of native poultry breeds that was previously established by the Ministry through Decree No. 19536 dated October 1, 2014, and subsequently entrusted to the Italian Breeders Association. The Book has been created with the aim of promoting conservation and valorization initiatives for native poultry breeds currently raised in the country.

In total, 52 breeds are currently admitted to it, distributed as follows: 22 belonging to the chicken species, 7 to the guinea fowl species, 3 to the Muscovy Duck species, one to the Muscovy Crested Duck species, 3 to the goose species, 8 to the turkey species, and 8 to the pigeon species.

In Table n.6 you can see the list of chicken and turkey breeds currently included in the registry.

Table 6 - Registry of Italian Chicken and Turkey Breeds

Chicken (*Gallus gallus*)		**Turkey (*Meleagris gallopavo*):**	
1	Ancona	**1**	Brianzolo
2	Bianca di Saluzzo	**2**	Bronzato comune
3	Bionda piemontese	**3**	Bronzato dei Colli Euganei
4	Collo Nudo italiana	**4**	Castano precoce
5	Ermellinata di Rovigo	**5**	Ermellinato di Rovigo
6	Livorno	**6**	Romagnolo
7	Mericanel della Brianza	**7**	Nero d'Italia
8	Millefiori Lonigo	**8**	Tacchino di Parma e Piacenza
9	Millefiori piemontese		
10	Modenese		
11	Mugellese		
12	Padovana		
13	Pepoi		
14	Pollo trentino		
15	Polverara		
16	Robusta lionata		
17	Robusta maculata		
18	Romagnolo		

19	Valdarnese bianca	
20	Valdarno	
21	Siciliana	
22	Tirolese o Tirolerhuhn	

These breeds represent an important part of the Italian poultry genetic heritage and are crucial for the conservation of biodiversity in the country's poultry sector. Scientific studies have been conducted to genetically characterise these breeds and gain a better understanding of their genetic diversity. Additionally, studies have been carried out on breeding, production performance, product quality, animal welfare, and physiological characteristics of many of these breeds.

The preservation of Italian native poultry breeds represents a crucial priority for agriculture and biodiversity conservation in Italy. While significant progress has been made in this field, it is imperative to maintain and intensify efforts to ensure the survival and prosperity of these breeds in the future. The conservation of native poultry breeds not only contributes to genetic biodiversity but also holds profound cultural and socioeconomic value. This is a commitment that involves farmers, breeders, institutions, and society, with the goal of ensuring a sustainable future for these unique and precious breeds.

Part.2

Overview of animal genetic diversity conservation with a special emphasis on *ex situ in vitro* conservation strategy

4. Animal biodiversity conservation strategies

Biodiversity conservation is essential to maintain the stability of ecosystems and includes the preservation of ecosystems, habitats, species, and genes. Central to this conservation effort is the 'chain mechanism', a fundamental concept that emphasises the interconnection between gene conservation and the protection of all populations of a species. This approach emphasises the critical need to preserve habitats and ecosystems, recognising them as indispensable resources for the survival and prosperity of diverse species.

The commitment to preserve animal biodiversity has garnered substantial support from various international organisations (Arlidge et al., 2018). The core of animal conservation lies in maintaining genetic diversity within populations sufficiently large to ensure long-term sustainability. Unfortunately, due to both human activities and natural factors, many domestic and wild populations are small and fragmented, lacking opportunities for genetic exchange. This scenario leads to increased homozygosity and inbreeding, diminishing adaptability to environmental changes and causing fertility issues (Wildt et al., 2010), thereby elevating the risk of extinction.

In this context, it is crucial to develop conservation plans to preserve genetic resources globally. In addition to habitat protection, two conservation strategies are considered (Bermejo et al., 2019):

1. *In Situ* Conservation*:* This involves maintaining animal populations in their natural environment, ensuring enough to preserve biodiversity and ensure survival.
2. *Ex Situ* Conservation: is a biodiversity preservation strategy that entails the management and protection of populations of organisms outside their original natural environment. *Ex situ* conservation can take place *in vivo* or *in vitro.*

In situ and *ex situ* strategies have different methods of biodiversity conservation. *In situ* conservation is favoured as it enables genetic resources to undergo a natural evolutionary path through selection processes within their native environment. This approach aligns with the preservation of species within their natural habitats. However, it's important to note that not all species can be effectively safeguarded in their original environments. When *in situ* conservation fails to maintain genetic diversity or prevent species extinction, it is prudent to consider *ex situ* conservation. These two conservation strategies are complementary and should be used synergistically to create an integrated conservation strategy. The primary goal is biodiversity protection through in-situ conservation, and

ex situ conservation plays a vital role by providing material that can potentially be reintroduced into the natural environment.

4.1 Ex situ cryopreservation of germplasm

Ex situ conservation involves the maintenance and reproduction of endangered animals under partially or fully controlled conditions in specific areas. Specimens of a species, subspecies, or variety are collected and preserved in genetic banks, botanical gardens, zoos, or in the form of seeds, ova, tubers, tissue explants, pollen, or DNA preserved under special artificial conditions. *Ex situ* conservation can be carried out both *in vivo* and *in vitro*. In *in vivo* strategies living individuals of the population are typically conserved outside their natural distribution areas, such as in parks, zoos, or research centers. However, the use of an *ex situ-in vivo* strategy to preserve genetic diversity can be ineffective due to issues such as reproductive failure due to disruption of social dynamics and natural mating patterns. This can also increase the risk of inbreeding, resulting in reduced fertility. Conservation of live animals entails risks of unexpected diseases and high costs for food and housing. Additionally, animal transportation poses challenges related to welfare, costs, and disease transmission. *Ex situ in vitro* conservation involves the preservation of plant tissues, including cells, tissues, or parts of plants, within a laboratory environment. Therefore, *ex situ* cryopreservation of germplasm, which is a long-term survival strategy, is considered an efficient approach for genetic conservation. This practice is essential for biodiversity conservation, involving the preservation of genetic material in various formats such as sperm, ova, embryos, somatic cells, and DNA sequences. These valuable materials are stored in specialised facilities such as germplasm banks or cryobanks, typically located in research institutes, universities, government agencies, or private companies. The *ex situ in vitro* conservation technique involves the long-term storage of reproductive cells or vital tissues in liquid nitrogen at -196°C. These cryopreserved materials can be used to recreate offspring through assisted reproductive technology (ART), which includes artificial insemination (AI), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), embryo transfer (ET), and primordial germ cell transplantation (PGC). Although not applicable to all species, cryopreservation of somatic cells and induced pluripotent stem cells (iPSCs) is another approach to achieve the same goal. These techniques are widely used in various species. However, optimizing the species-specific protocol is necessary to achieve successful results.

4.1.1 Cryopreservation methods

In general, two freezing techniques can be distinguished: slow freezing and vitrification. Slow freezing, also known as conventional freezing method, involves a controlled and gradual procedure of temperature decrease, which is associated with the risk of ice crystallisation that can cause damage

to cell membranes. It is achieved by exposing cells to liquid nitrogen (LN_2) vapors, at various heights above the LN_2 surface, or using a programmable freezer for a gradual temperature decrease. Still, biological material must be cooled at a series of cooling rates that are fast enough to prevent excessive dehydration but slow enough to allow sufficient cell dehydration to prevent intracellular ice formation. Additionally, this system uses a relatively low concentration of cryoprotectants (CPA). "Vitrification" is an ultra-rapid cooling method, where cells are directly immersed in liquid nitrogen. This method commonly requires a high concentration of CPA to be added before the cooling phase to reduce water content and avoid the risk of intracellular ice formation; in this case, the entire sample will vitrify. Yet, the concentration of CPA in vitrification solutions can be minimized by using very high cooling and thawing rates, up to complete absence of CPA in case of extremely high cooling rates. Modern genetic diversity conservation technologies offer various options for the cryopreservation of germplasm and tissues, including sperm, oocytes, embryos, and somatic cells, among others. However, the applicability of these options varies depending on the species, and there is limited knowledge available on their germplasm reproduction and cryobiology.

4.1.2 Advantages and disadvantages of sperm cryopreservation

Compared to other germ cells, sperm is relatively easier to collect in large quantities (Comizzoli & Wildt, 2013; Santiago-Moreno & Blesbois, 2022) since spermatozoa are continuously produced by males from sexual maturity until the end of their life, allowing for massive individual collection (Bolton et al., 2022). In addition to ejaculates, sperm can also be collected from mature individuals within 48 hours of animal death or castration. This technique can be achieved by washing sperm from the vas deferens in birds (Villaverde-Morcillo et al., 2016) or from the epididymis in mammals (Martínez-Fresneda et al., 2019; Galarza et al., 2021). Due to the unique characteristics of sperm in each species, protocols for sperm cryopreservation should be customized to fit the specific species (Leibo & Songsasen, 2002; Comizzoli, 2015). However, the general phases of the method remain the same across all species. This process involves several stages: semen collection, addition of cryoprotectants (CPA), cellular dehydration, freezing, storage in liquid nitrogen, and thawing before use (Ugur et al., 2019). However, the selection of CPAs and their dosage may vary among different species, depending on the specific requirements of the sperm and tolerance levels (Schiewe et al., 1991; Stoops et al., 2007; Zee et al., 2008; Robeck et al., 2011). Furthermore, cooling rate and container material are other factors that need to be tested (Comizzoli & Wildt, 2013). In mammals, the male is heterogametic, producing sperm carrying both X and Y chromosomes. Cryopreservation of mammalian sperm allows for the preservation of the entire nuclear genome and the restoration of the same genetic material after thawing. However, the male avian is homogametic (ZZ) and produces only Z sperm. Consequently, sperm cryopreservation alone does not allow for the preservation of the

entire genome in birds. To achieve this goal, it is necessary to combine sperm cryopreservation with other germplasm cryopreservation techniques, such as preservation of PGCs.

4.1.3 Cryopreservation of oocytes and embryos

The cryopreservation of oocytes and embryos plays a crucial role in the dissemination and conservation of animal genetic resources. However, both oocytes and embryos are complex cellular aggregates rich in water and highly sensitive to cooling and cryopreservation. Despite recent advancements, the optimal protocol remains to be defined. Additionally, the freezing of oocytes in avian and fish species has not yet yielded satisfactory results. This difficulty is primarily attributed to their large sizes, high lipid content, and polar structure of the eggs of these species. Moreover, the membranes of avian and fish oocytes exhibit variable permeability to cryoprotective agents (CPAs), which can lead to increased ice crystal formation during freezing and subsequent cellular structural damage.

Vitrification, involving the use of a high osmolarity solution containing a high concentration of CPAs and rapid immersion of samples directly into liquid nitrogen, has been found to be more effective than conventional slow freezing methods for oocytes and embryos. Due to the high viscosity of the vitrification medium and rapid cooling rate, a glass-like state is quickly achieved, preventing ice crystallization and minimizing structural damage. However, during vitrification, intracellular ice can still form if the CPA concentration is not sufficiently high. This presents a dilemma, as high CPA concentrations can be toxic to cells. Consequently, a more recent strategy called ultra-rapid vitrification has been developed, aiming to minimize cryo-injury by increasing cooling and warming rates. This approach allows for the use of lower CPA concentrations, thus reducing toxicity to oocytes and embryos. Various specific devices, such as the pulled straw, cryoloop, nylon mesh piece, and gel-loading tip, have been developed to achieve higher cooling and warming rates.

While embryo production, cryopreservation methods, and transfer are well-established in some species such as ruminants and rodents, further improvements are needed in other species due to significant differences in embryonic structure and molecular contents. In general, unfertilized oocytes are more sensitive to cryo-injury than embryos, and in vitro-produced embryos are more susceptible to cryopreservation than those derived *in vivo*.

4.14 Cryopreservation of Somatic Cells and Stem cell

Somatic cells can serve as a prudent backup in circumstances where cryopreservation of gametes and embryos is not financially or technically feasible or has very low success rates. In its simplest application, somatic cell preservation requires only the collection and direct freezing of a piece of tissue. This means that obtaining somatic cell collections is easy and inexpensive, making them a

highly attractive option for genetic conservation, especially for countries with many breeds and/or limited resources. However, the complexity and costs associated with using somatic cells are much greater than those for other types of cryopreserved material. Manipulating somatic cells involves cloning and culturing after thawing (or prior to freezing), reprogramming nuclei, and collecting oocytes by ovum pick-up or from slaughtered animals, making it an extremely costly technique that requires high levels of infrastructure and technical expertise. Donor somatic cells can be cryopreserved using cryoprotective agents (CPAs) (Dai et al., 2006), without CPAs (Hoshino & Saeki, 2010), or even desiccated (Wakayama et al., 2022) before conducting somatic cell nuclear transfer (SCNT) technology. SCNT is an advanced biotechnology used to produce genetic replicas of an individual, also known as clones. The first animal obtained through SCNT was the famous sheep Dolly (Campbell et al., 1996), produced by transferring the nucleus from a donor somatic cell into the cytoplasm of an enucleated oocyte. After being artificially activated, the oocytes reprogram the exogenous donor nucleus through dedifferentiation and develop into early embryos in vitro. These embryos can then be successfully transferred into the uterus of a suitable recipient and implanted. SCNT has potential for various purposes, such as rescuing endangered species, reviving extinct species, producing transgenic animals, and cloning farm animals with desirable phenotypes (Oback, 2008; Iqbal et al., 2021). However, offspring survival rates are generally very low (Chavatte-Palmer et al., 2013). Currently, this technology has been used for many domestic species, including cattle, goats, pigs, sheep, water buffaloes, horses, mules, camels, deer, and rabbits. In this context, if the production of live animals from somatic cells were to develop to a point where it becomes both reliable and economical, somatic cell preservation would become an attractive option for cryopreservation of genetic resources.

4.1.5 Other germoplasm

Recent studies have explored the potential of transplanting spermatogonial stem cells (SSCs) or testicular or ovarian tissue after cryopreservation. Spermatogonia reside in the basal layer of the seminiferous tubules of the testis and have the ability to generate spermatozoa. Isolated cases of successful transplantation of spermatogonial stem cells in mice (Gouk et al., 2011) and fish (Okutsu et al., 2006; Morita et al., 2012), and of testicular tissue in bulls (Herrid et al., 2006) and chickens, (Song and Silversides, 2007); have been reported, with both techniques producing viable spermatozoa. The transfer of spermatogonia could potentially be used to pass genetic material from one generation to another, in combination with cryopreservation, providing a way to conserve the genes of male animals for future generations. The isolation of spermatogonial stem cells (SSCs) from cryopreserved testicular tissues and their transplantation into surrogate males is a potential method for generating viable sperm for fertilization. A more recent technique, called induced pluripotent stem

cells(iPSCs), involves generating stem cells directly from somatic cells through genetic reprogramming and transfection with pluripotency-related genes. These iPSCs can be differentiated in vitro into spermatozoa or oocytes for subsequent fertilization. With this approach, it is crucial to select vital tissues that can be reprogrammed for iPSC generation after cryopreservation. Fibroblasts are widely used for this purpose.

Among the various techniques used, nuclear DNA storage is useful for research purposes, despite the challenges encountered in reproducing some of them. However, it is currently not employed for the restoration of live animals. Recently, attention has shifted to alternative forms of germplasm conservation, particularly primordial germ cells (PGCs) for these species. PGCs are precursor cells for both oocytes and spermatozoa, formed during early embryonic development. In fish and chickens, these cells undergo rapid proliferation after migration to the gonadal ridge through the bloodstream. Some studies have shown that functional spermatozoa and oocytes can be obtained through the transplantation of male or female PGCs in fish (Kobayashi et al., 2007), even in endangered species, and in birds, (Ono et al., 1996; Kim et al., 2005; Kang et al., 2008), producing live offspring. These results indicate promising potential for future cryopreservation systems, although this technology requires further development.

4.2 Cryobank: Preservation of Animal Species with Ex Situ in Vitro Technology

In recent years, increased awareness of the importance of genetic resources has generated growing interest and actions aimed at their preservation and valorisation. Consultative forums have been established at both European and international levels within the European Union, the European Association for Animal Production (EAAP), and the Food and Agriculture Organisation (FAO) of the United Nations to discuss this crucial issue and initiate actions aimed at preserving global genetic resources (Daunchin-Burge et al., 2002).

Although the practice of cryopreservation of animal germplasm is not new, it has become significantly effective since the late 1950s with the cryopreservation of bovine semen. Over time, cryopreservation techniques have been refined, expanding the ability to preserve semen from various species and a wide range of tissues, including embryos, blood cells, fibroblastic cells, and primordial germ cells, for livestock species of interest (Amstislavskii and Trukshin, 2010).

However, despite progress, cryobanks of germplasm and animal tissue remain underutilised in national conservation programmes. Someone feels that the approach is too expensive or requires advanced technical skills. One of the main objectives of a germplasm repository is the possibility of recreating breeds or reproductive lines in case of disaster losses (Comizzoli et al., 2010). Furthermore, cryopreserved material can support *in vivo* conservation by reducing inbreeding and genetic drift in

small managed populations, combining live animals and cryopreserved germplasm as a powerful tool for conservation (Meuwissen, 2007).

The resources of genetic banks can serve as reserves to address potential emerging genetic issues, such as the decrease in effective population size (Ne) and the increase in inbreeding, which can promote the frequency of deleterious alleles not apparent in larger populations (Sonesson et al., 2002; Woelders et al., 2006).

Another important use of cryopreserved material is the ability to develop new lines or breeds, or to rapidly modify the evolution or selection of the population. For instance, the conservation of original or extreme genotypes can quickly steer the genetic trend of a selected population (Verrier et al., 2003).

It should be emphasized that these forms of *ex situ* conservation should integrate with *in situ* conservation, collaborating with conservation programs and contributing to the safeguarding of endangered species.

Finally, genetic banks can serve as a primary source of material for researchers conducting DNA research. Preserving DNA samples alongside germplasm can expedite researchers' access to samples and provide access to common groups of animals for genotyping research. Additionally, genetic banks can provide multi-generational samples that can be extremely useful in such studies, allowing for the study of genetic variation over time and across generations. Currently, there are no international or European regulations for the organization of a germplasm bank. However, guidelines are available from the FAO titled "Cryopreservation of Animal Genetic Resources" (FAO, 2012).

Before initiating the collection of material to be preserved in a gene bank, it is essential to assess the priority breeds/species for *ex situ in vitro* conservation. The decision on which breeds to conserve should involve the National Advisory Committee on Agricultural and Genetic Resources (AnGR) (FAO, 2011) or a similar committee, in consultation with the stakeholder community. Various quantitative procedures are used to determine conservation priorities, considering both the risk of extinction and conservation value (Ruane, 2000).

The risk of extinction is assessed based on current and future population sizes and geographic distribution. Animal census and breed surveys are crucial for this assessment. Breeds at higher risk receive top priority for conservation efforts.

Conservation value depends on genetic diversity and phenotypic traits, such as environmental adaptability and unique characteristics. Breeds that provide important environmental services or have cultural value are considered for conservation. According to the FAO classification (2003), breeds are divided into 7 risk categories: extinct, critical, critical-maintained, endangered, endangered-maintained, not at risk, and unknown status.

To establish a cryobank, in addition to risk assessment, phenotypic and genotypic characterization is essential. This process identifies breeds/species based on their external and genetic characteristics, facilitating conservation program planning. In recent years, single nucleotide polymorphism (SNP) genetic markers have been replacing microsatellites due to their greater reliability in genetic information, making them ideal for long-term conservation efforts (Roques et al., 2019).

An essential aspect to consider in establishing a cryobank is the required space, which can vary significantly and depends on the quantities of germplasm to be preserved. This, in turn, is influenced by the goals of the gene bank, the diversity of species and breeds to be conserved, and the financial resources available for the conservation program. Regardless of size, certain features are crucial to ensure the proper functioning of the cryobank, including physical infrastructure (such as the building and other structures), durable equipment, security measures, centralization, accessibility, and the presence of specialized personnel.

A cryostorage facility requires three main areas: (1) an area for germplasm collection, (2) a working area to facilitate sample handling and germplasm freezing, and (3) an area to accommodate liquid nitrogen storage tanks, where the cryopreserved material is located (Ryan et al., 2016; FAO, 2012). Although all these activities can be conducted at the same location, each requires separate facilities.

During the initial phase, animals are moved to the cryostorage facility using suitable facilities to house the animals themselves. This makes the germplasm collection process more practical and manageable. In the case of semen donors, animal transfer also allows for their training for collection, a crucial aspect to ensure an increase in the yield and quality of collected semen.

Part. 3

Notes on the spermatozoa of fish and poultry

5. Specific feature of teleost fish semen

Fish sperm often have a very simple structure that is often characterised by a very simple structure. It consists of four main portions: the head, which contains the nucleus; the neck, which encloses the mitochondria and centrioles; the middle piece, in which numerous mitochondria form a long spiral around the flagellar filament; and the tail, which is endowed with motility and ensures the sperm's movement (Figure n 6).

In teleost fish, it is typical for spermatozoa to lack an acrosome (in contrast to chondrostean such as sturgeon). Their outer membrane, known as the impenetrable chorion, has a small opening called a micropyle that allows access to the oocyte membrane. Fish sperm typically have a spherical nucleus with highly condensed and uniform chromatin and a nuclear fossa. They also have an intermediate piece of variable size that may or may not have a cytoplasmic channel. Finally, it is common for them to have one or two flagella of considerable length (Leung and Jamieson, 1991).

The paternal genetic material is mostly found in the nucleus of the head. With a diameter of 2-4 m, the heads of most fish species are almost spherical or oval in shape. There are some cases where the head shape is elongated, reaching lengths of up to 9 m and widths of 2 m, such as in sturgeon, paddlefish and eel sperm (Gibbons et al., 1983; Linhartova et al., 2013). Centrioles and mitochondria, which typically number between 2 and 9 per sperm and produce ATP to power motility, make up the majority of the intermediate component (Cosson, 2012).

The plasma membrane surrounding the head of the spermatozoa is tightly attached to the nucleus, leaving only a thin layer of cytoplasm between them. In the middle, some folds in the plasma membrane along the root of the axoneme create overlapping membrane layers. In some species, such as Salmonidae and Percidae, the plasma membrane surrounding the axoneme has paired lateral extensions resembling a helical fin extending along the tail. These lateral ribbons are not observed in other species such as *Labridae*, while in *Sparidae* either 2, 1 or no lateral ribbons can be found depending on the species (Lahnsteiner and Patzner, 2008).

The sperm plasma membrane plays a crucial role in activating motility. It is through this membrane that the ionic changes responsible for initiating flagellar beating are sensed when the sperm are released into the water. Several ion channels are present in the plasma membrane of fish sperm, and progestin receptors have been identified in the spotted seatrout (*Cynoscion nebulosus*) that contribute to motility (Tubbs and Thomas, 2008).

Since teleost sperm lack an acrosome, the plasma membrane is also essential for gamete fusion. For example, in some species, such as rainbow trout, the ganglioside GM3 located in the sperm head is involved in sperm-egg binding (Yu et al., 2002). Some uncharacterised proteins in the head region have also been implicated in the fertilisation process (Beck et al., 1992).

From a biophysical standpoint, the lipids in the plasma membrane influence its fluidity, and both proteins and lipids contribute to its permeability to water and ions. In rainbow trout sperm, membrane lipids have been extensively studied, with a molar ratio of cholesterol to phospholipids ranging between 0.4 and 0.6. Phosphatidylcholine makes up 50% of total phospholipids, primarily in the outer leaflet, while phosphatidylserine (10%) and phosphatidylethanolamine (30%) are concentrated in the inner leaflet. A polyunsaturated fatty acid, 22:6 n-3, is abundant (over 10% of total fatty acids), contributing to a high ratio of unsaturated to saturated fatty acids, which reaches 1.30. These lipid characteristics influence the plasma membrane's permeability to water, suggesting the absence of aquaporins that would facilitate water penetration (Labbé and Maisse, 2001).

The basal body of the flagellum anchors it to the sperm head and is formed by the centriolar complex of the intermediate component. Salmon sperm are flagellated, approximately 32-39 μm in size, with a concentration of up to 20 billion sperm per ml of seminal fluid (Iaffaldano et al., 2016b). The length of fish sperm flagella varies from 20 to 100 μm, and the axoneme, a cylindrical microtubule network, induces flagellar bending (Inaba, 2003). The typical "9 + 2" axoneme has a central pair of single microtubules and nine pairs of peripheral doublet microtubules, but some fish families, such as Anguilliformes and Elopiformes, exhibit a "9 + 0" pattern without central microtubules (Gibbons et al., 1983). Structural connections between the peripheral doublet microtubules and the central pair are made by radial spokes. In the cytoskeleton, studies have focused on the flagellar axoneme, which in most fish species normally has nine pairs of peripheral microtubules and one central pair.

However, in species such as eel, central microtubules can be absent (Gibbons et al., 1983). Flagellum bending results from sliding microtubule doublets mediated by dynein arms, with actin closely associated with the plasma membrane.

Nuclear organisation in fish sperm varies; for example, rainbow trout have an elongated nucleus with an invagination covering about a third of its length, while other species may differ (Billard, 1983; Ohta et al., 1993; Lahnsteiner et al., 1998). The protein composition of the nucleus varies, affecting chromatin condensation and sperm DNA stability (Munoz-Guerra et al., 1982; Saperas et al., 1993; Avramova et al., 1983). Fish sperm can be categorised as aquasperm (adapted to the external aquatic environment) or introsperm (adapted to the internal environment) (Leung and Jamieson, 1991). In teleosts with external fertilisation, the primary activity of sperm is motility, which is initiated upon release into the aquatic environment. Activation signals influenced by the aquatic environment

include osmotic pressure, ionic and gaseous components, and egg-derived substances for sperm orientation (Cosson et al., 2019). Sperm motility in freshwater species lasts 0.5-2 minutes, and cryopreservation diluents preserve vitality for later use (Cosson, 2010).

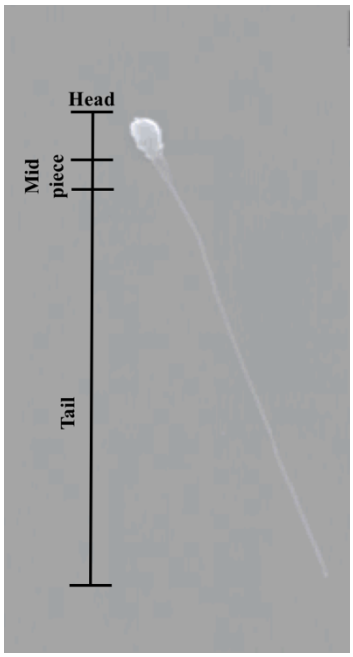


Figure 6. Microscopic view of teleost fish spermatozoa

5.1 Female Gametes, Ovarian Fluid and Effects of Ovarian Fluid on the Performance of Freshwater Fish Sperm

In teleost fish, oogenesis can be divided into five stages: previtellogenic, lipid yolk, protein yolk, fully grown oocyte, and mature oocyte (Berois et al., 2004). The oocyte develops within the ovarian follicle, an organized structure comprising the oocyte surrounded by granulosa cells, theca cells, and an outer basal lamina (Wallace and Selman, 1981). All vertebrate species have an oocyte covered by the vitelline envelope, which is also known as the eggshell, zona pellucida, radiata zona, or chorion. However, in teleosts, the interaction with sperm occurs exclusively at the micropyle level. This funnel-shaped structure serves as the only entry point for sperm and forms during the deposition of the egg envelope (Berois et al. 2011). The vitelline envelope has several functions, including protecting the growing oocyte and developing embryo, absorbing nutrients and other molecules, and guiding sperm towards the micropyle (Dumont and Brummet, 1980). Following the entry of sperm into the micropyle, it fuses with the oocyte's cell membrane and other structures (microvilli), thus resulting in fertilisation (Brummett and Dumont, 1979; Dumont and Brummett, 1980; Hart and Donovan, 1983). As mentioned earlier, since fertilisation in Mediterranean trout is external, the female will simultaneously release eggs and ovarian fluid into the surrounding deposition

environment, where several males will concurrently release their seminal fluid, allowing sperm to come into contact and interact with the eggs for fertilisation.

The ovarian fluid (OF) is a biological fluid of maternal origin, clear in color (Satia et al., 1974), released together with the eggs during deposition. Its exact origin remains largely unknown; however, it appears to be produced through the secretion of epithelial cells in the ovarian cavity, related to the metabolic activity of follicular cells and filtration from the bloodstream (Yamamoto, 1963; Lahnsteiner et al., 1996). During ovulation, the epithelial cells and the tunica albuginea of ovarian follicles enlarge and become columnar, leading to the accumulation of ovarian fluid in the ovarian lumens (Lahnsteiner et al., 1996). The composition of OF varies both between species and within the same species, and its physical properties (color, volume, viscosity) generally reflect the chemical characteristics (pH, osmolarity) and biochemical composition.

The color is typically clear (Satia et al., 1974), and the volume has not been quantified for many species. However, according to some studies conducted on salmonids, it has been shown that ovarian fluid can constitute up to 10-30% of the total egg mass (Lahnsteiner et al., 1999; Wojtczak et al., 2007). The viscosity of ovarian fluid, on the other hand, varies significantly among females depending on the maturation stage of the eggs (Lam et al., 1978; Roufidou, 2017) and is typically two to three times higher than that of water.

From a chemical perspective, a common characteristic for most fish is an alkaline pH, which helps stabilize the microenvironment around the egg, especially in acidic waters. However, this parameter can also vary both interspecifically (pH ranging from 6.2 to 8.8) and intraspecifically, as is the case with rainbow trout (pH ranging from 7.3 to 8.5; Lahnsteiner et al., 1996)

The second chemical parameter that characterizes ovarian fluid is osmolarity, which is lower in freshwater fish (190 to 322 mOsmol/kg) compared to marine species (289 to 514), and like pH, it can vary among individuals (Zadmajid et al., 2019) and during the breeding season (Rosengrave et al., 2009a). Low osmolarity values can result from the influx of water into the celomic cavity during stripping or from variations in ovarian secretions (Aegerter and Jalabert, 2004).

Examining the biochemical properties of ovarian fluid, in salmonids, the concentration of electrolytes is similar, but not identical, to that of blood plasma, and it also contains various nutrients, metabolites, and hormones. The main ions found in ovarian fluid in most fish species are chloride (Cl^-, ranging from 94.0 to 172.7 mmol/L) and sodium (Na^+, ranging from 104.7 to 213.7 mmol/L), in addition to calcium (Ca^{2+}) and magnesium (Mg^{2+}), present in quantities less than 3.8 mmol/L, and potassium (K^+) with values ranging from 1.7 to 19.3 mmol/L depending on the species.

Protein levels in ovarian fluid have been detected at high concentrations, showing intra-species variations (117.3–669.8 mg/100 mL for rainbow trout) and inter-species variations (4.8–1331.1

mg/100 mL; Zadmajid et al., 2019). Currently, in salmonids, up to 174 proteins have been identified (Johnson et al., 2014; Nynca et al., 2015), involved in lipid binding and metabolism, carbohydrate and ion transport, innate immunity, maturation and ovulation processes, and the ability of male gametes to fertilize eggs (Nynca et al., 2015; Lahnsteiner, 2002).

It has been demonstrated that ovarian fluid influences various aspects of sperm, including activation, chemotaxis, longevity, swimming performance, and swim trajectory (Rosengrave et al., 2009a; Rosengrave et al., 2009b; Gasparini et al., 2012; Gasparini and Evans 2013; Liberti et al., 2016; Zadmajid et al., 2019).

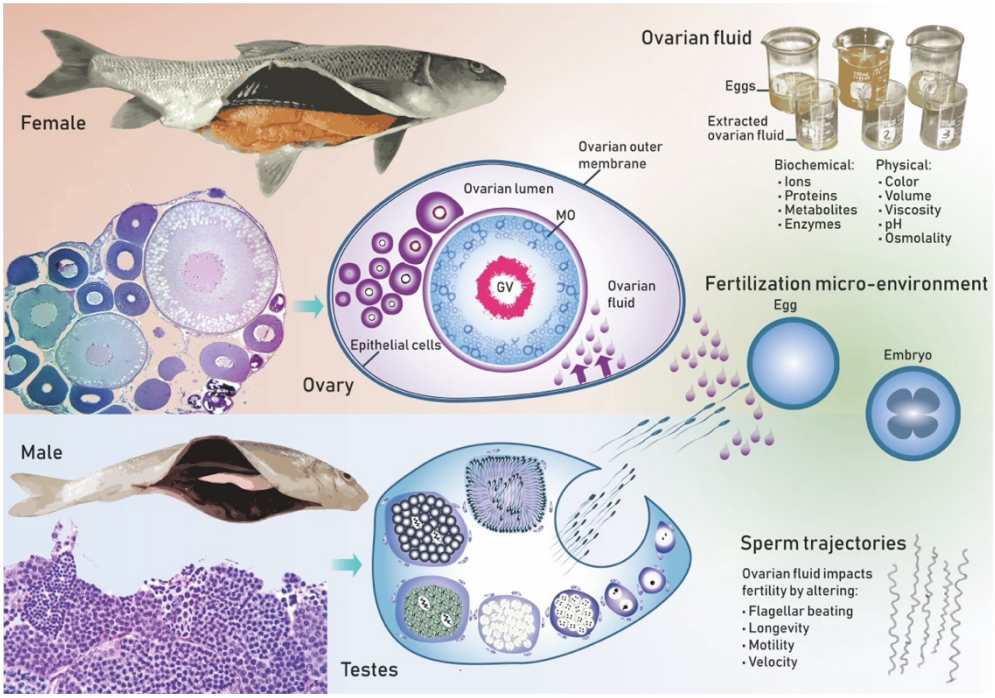


Figure 7 - Schematic illustrating gamete development and fertility in externally fertilizing fish. Maternal development is in the upper section, while paternal development is in the lower section, each depicted with a histological image and cross-section of the mature gonad. each female creates a unique fertilization micro-environment by expelling distinct ovarian fluid with an egg batch (top right). ovarian fluid can directly impact fertilization outcomes by modifying sperm trajectories (bottom right). abbreviations: gv: germinal vesicle; mo: mature oocyte. *(Zadmajid V. etl., 2019)*

Sperm motility and velocity are fundamental factors for reproductive success, as they enable sperm to reach the micropyle more quickly (Alavi et al., 2019). In some salmonid species, it has been observed that ovarian fluid has a positive effect on the percentage, speed, and duration of sperm motility. For example, a study on *Oncorhynchus mykiss* demonstrated that increasing concentrations of ovarian fluid increased the number of high-velocity sperm and improved their straight-line swimming and oscillation (Kanuga et al., 2012). Additionally, in *Syngnathus abaster*, ovarian fluid extended the longevity of sperm (Dzyuba et al., 2008). In the species *Oncorhynchus tshawytscha*, reducing the viscosity of ovarian fluid compensated for the inhibitory effects of a more viscous swimming medium for sperm (Rosengrave et al., 2009a).

While not yet fully understood, it is hypothesised that the biochemical components of ovarian fluid improve the efficiency of sperm movement. However, the exact mechanism remains largely unknown. Additionally, it appears that the interactions between ovarian fluid and sperm are more pronounced at higher concentrations of ovarian fluid (Butts et al., 2017).

Fish ovarian fluid contains a range of organic and inorganic compounds that can affect the reproductive microenvironment and, thus, the swimming characteristics of sperm and their ability to fertilise. Among the inorganic compounds, it has been observed that different concentrations of ions present in ovarian fluid have a significant impact on sperm performance. For example, in the case of the fish *Gadus morhua,* it has been found that calcium (Ca_2^+) has a negative correlation with sperm motility, speed, and oscillation index. In other words, higher concentrations of calcium ions in ovarian fluid seem to have an unfavourable effect on the sperm's ability to move efficiently and exhibit oscillatory motion, which are important for successfully reaching and fertilising the egg (Beirão et al., 2015) These results suggest that the chemical composition of ovarian fluid, particularly the presence of specific ions like calcium, can significantly influence sperm performance and, consequently, the effectiveness of the reproductive process in fish.

In the OF fish, inorganic ions can influence sperm performance in various ways. For example, in *Oncorhynchus tshawytscha*, sperm longevity was found to be negatively correlated with calcium (Ca^{2+}) and magnesium (Mg^{2+}) concentrations, while the percentage of motile sperm appeared to be positively correlated with magnesium (Mg^{2+}) concentration (Rosengrave et al., 2009a). These findings highlight the complex and species-specific interactions between the chemical composition of ovarian fluid and sperm behavior in different fish species.

In *Oncorhynchus mykiss*, potassium ions (K^+), calcium ions (Ca^{2+}), and magnesium ions (Mg^{2+}), which are present in high concentrations in ovarian fluid, did not activate the sperm, while sodium ions (Na^+), present in lower quantities, were positively correlated with sperm motility activation. Specifically, high levels of potassium ions (K^+) in ovarian fluid were found to inhibit sperm motility in salmonids (Inanan and Öğrement, 2015).

Among the biochemical factors, proteins in ovarian fluid also appear to influence sperm performance. For example, in the fish *Gadus morhua*, elevated protein levels were negatively correlated with sperm velocity. Some specific proteins in ovarian fluid, although not fully identified, may influence sperm velocity in relation to mate choice (Beirão et al., 2015). These findings illustrate the intricate interplay between the chemical and biochemical components of ovarian fluid and their effects on sperm behavior in different fish species.

Indeed, among the "non-chemical" factors, both the pH and osmolarity of ovarian fluid appear to play a crucial role in influencing sperm performance in fish. These factors can have a significant impact

on sperm motility and function during the reproductive process (Wojtczak et al., 2007; Dietrich et al., 2007; Diogo et al., 2010; Inanan and Öğrement, 2015). These non-chemical factors, in conjunction with the chemical and biochemical components of ovarian fluid, contribute to the complex and species-specific interactions that influence the reproductive success of fish.

In addition, it has been observed that ovarian fluids from different females can have varying effects on the sperm performance of different males, particularly in *Oncorhynchus mykiss* (Inanan and Öğrement, 2015). This further highlights the complexity of the interactions between ovarian fluid and sperm, as well as the potential for individual variation in how these interactions affect reproductive success in fish. Some samples of ovarian fluid inhibited sperm motility, while others activated and influenced it differently. These results indicate that the composition of ovarian fluid can influence females' cryptic choice, directing sperm competition towards specific male phenotypes that will offer benefits to the offspring.

Furthermore, studies have been conducted to assess how ovarian fluid may have different effects on sperm performance based on genetic relatedness, phenotypic relatedness, and the geographic origin of individuals involved in reproduction. These studies contribute to a better understanding of how variability in ovarian fluid can influence reproductive success and the adaptation of fish species (Urbach et al., 2005; Neff et al., 2005; Rosengrave et al., 2016a; Alonzo et al., 2016; Lehnert et al., 2017). In some fish species, sperm activity can be enhanced when activated in the ovarian fluid of either related or unrelated females (Gasparini and Pilastro 2011; Butts et al., 2012; Lehnert et al., 2017). For example, in the case of *Salvelinus namaycush*, the velocity of sperm activated in the ovarian fluid of related females was significantly higher than that of unrelated males. This phenomenon could represent part of a female-triggered recognition system to select sperm from a specific genotype (Butts et al., 2012). In the species *Oncorhynchus tshawytscha*, males less related to females had higher sperm velocity in ovarian fluid compared to related males, suggesting that certain genes in the female genome may influence sperm competition by favoring certain individuals (Geßner et al., 2017).

The ovarian fluid has increased both the number of spermatozoa towards the micropyle and their longevity in the species *Salmo salar* and *Salmo trutta*. These results indicate how cryptic choice can promote reproductive isolation through a specific chemotactic influence of ovarian fluid on spermatozoa swimming behavior (Yeates et al., 2013).

In the species *Symphodus ocellatus* and *Oncorhynchus tshawytscha*, ovarian fluid has positively influenced sperm competition towards males with specific parental phenotype, improving sperm speed and motility (Alonzo et al., 2016; Lehnert et al., 2017). Furthermore, it has been observed that ovarian fluid from females of *Gadus morhua* from a southern population had a greater inhibitory

effect on the sperm of males of northern origin compared to those activated in ovarian fluids from females of the same population. Analysis suggested that the difference could be due to the concentration of Ca^{2+} in the ovarian fluids (Beirão et al., 2015). Despite numerous studies conducted to understand this phenomenon in species with external fertilization, the underlying mechanism remains unknown, and interactions between ovarian fluid and sperm influenced by parental genotypes are still the subject of ongoing research. However, some authors have suggested that ovarian fluid and gamete recognition proteins, such as bindin-EBR1 and lysin-VERL, may influence fertilization by favoring genetically compatible males (Evans et al., 2013). Other authors have reported that signaling peptides in ovarian fluids and the sperm membrane can influence sperm competition and sperm behavior (Skarstein et al., 2005; Wood et al., 2007; Johnson et al., 2014).

In summary, from the results reported in the literature, it emerges that ovarian fluid plays a crucial role in influencing sperm performance, thereby contributing to female cryptic choice and sperm competition. These selective mechanisms appear to have evolved over time as a form of "reproductive isolation" to reduce hybridization between divergent populations and promote specific male genotypes or phenotypes that benefit offspring (Neff and Pitcher, 2005; Rosengrave et al., 2016a; Lehnert et al., 2017).

6. Specific characteristics of poultry semen

The male reproductive system in birds consists of a pair of testes, epididymis, deferent ducts (Figure 8 A and B), and accessory reproductive organs (Vizcarra et al., 2022; Valerie, 2023). Among these are the paracloacal vascular bodies, the dorsal proctodeal gland, and lymphatic folds (Fujihara, 1992). Unlike mammalian testes, which require a temperature around 2-8°C below core body temperature to support normal spermatogenesis and testicular functions (Abd El-Emam et al., 2023), avian testes are located within the abdominal cavity (Deviche et al., 2011), at an internal body temperature of 40-41°C (Padhi et al., 2016). Under these heat conditions, their functions are not suppressed, and they efficiently produce spermatozoa (Słowińska et al., 2020).

The testes are composed of interstitial tissue and seminiferous tubules, which are respectively the sites of androgen production and spermatogenesis (Estermann et al., 2021). The epididymis is made up of a series of ducts that eventually empty into the vas deferens. The ducts within the epididymis include the rete testis, efferent ducts, connecting ducts, and the epididymal duct. The epididymal ducts and the deferent duct are collectively known as the ducts conveying sperm from the tesis (Figure 8 C). The seminiferous tubules connect with the rete testis at specific points along the interface between the testis and the epididymis (Vizcarra et al., 2022).

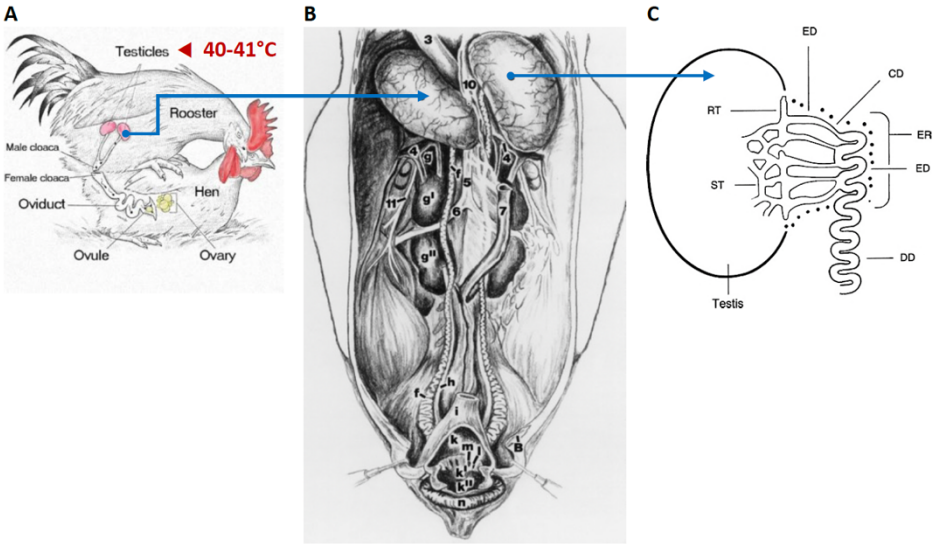


Figure 8 (A) A simple illustration of the reproductive anatomy in male and female chickens, as well as their mating behavior, known as the "cloacal kiss", (B) topography of the dorsal body wall of the rooster and (C) schematic of the excurrent ducts of the testis *(Vizcarra et al., 2022 and Valerie 2023).* abbreviations of (A): e-testis, f- deferent duct, h-ureter, i-opening of left deferent duct, m-opening of left ureter, (n) anus. abbreviations of (C): ST-seminiferous tubules, RT-rete testis, ED-efferent duct, CD-connecting duct, ER-epididymal region, ED-epididymal duct, DD-deferent duct.

Spermatogonia reside in the seminiferous tubules and undergo several distinct stages, known as spermatogenesis, to transform into active sperm. In the initial phase, spermatogonia cells proliferate and differentiate into primary spermatocytes through mitosis. This is followed by two consecutive divisions via meiosis, resulting in the formation of secondary spermatocytes and then haploid spermatids. The final stage is spermiogenesis, which primarily focuses on the morphological maturation of spherical spermatids into elongated ones. This process includes the condensation and elongation of the acrosome and nucleus, as well as flagellum development (Deviche et al., 2011; Haseeb et al., 2019). Apoptosis (Zakariah et al., 2022) and autophagy (Haseeb et al., 2019) are crucial processes for normal spermiogenesis, involving a dramatic loss of 97% of cytoplasm, with nuclear volume variations accounting for 96% of this reduction (Sprando and Russell, 1988). Mature spermatids are equipped with a head that contains an acrosome, a cylindrical nucleus, mitochondria, and a tail (flagellum). They are subsequently released by Sertoli cells into the seminiferous tubule lumen, a process known as spermiation, and subsequently transit into the epididymis (Vizcarra et al., 2022). The seminal plasma of chickens is derived from the proximal efferent ducts, epididymis, and deferent ducts (Santiago-Moreno et al., 2019) and is known to enhance sperm fertilization capacity. However, the precise molecular mechanisms behind this regulation have yet been fully elucidated (Santiago-Moreno and Blesbois, 2020). Furthermore, since avian sperm are capable of fertilizing the oocyte, unlike what is observed in mammals, the role of post-testicular maturation in avian sperm is still controversial (Nixon et al., 2014; Asano and Priyadarshana, 2022). Despite the fact that testicular spermatozoa are capable of binding to the perivitelline membrane and undergoing the acrosomal reaction, they do not exhibit normal motility (Ashizawa and Wishart, 1978; Nixon et al., 2014). This suggests that avian sperm fully acquire their motility capacities in the epididymis. This maturation process includes acrosin activation, acrosomal proteolytic activity (Thélie et al., 2019), chromatin compaction (Bernal et al., 2022), DNA protection (Santiago-Moreno et al., 2019), antioxidant capacity (Li et al., 2020), survival capacity during *in vitro* storage (Ahammad et al., 2011), and extension of sperm lifespan in the hen oviducts (Borziak et al., 2016). These results demonstrate the importance of post-testicular maturation for avian sperm, like mammals. However, the capacitation process, which occurs in mammals, has never been observed in avian sperm (Howarth, 1971). Additionally, avian sperm undergo an acrosomal reaction immediately after contact with the perivitelline membrane *in vitro* (Lemoine et al., 2008). This indicates that capacitation in the female genital tract is not essential for fertilization in birds.

At the time of ejaculation, avian spermatozoa exhibit characteristics common to all amniote species and specific adaptations to their complex internal fertilization system. They consist of slender, elongated gametes with a head, an intermediate portion, and a long flagellum (Figure 8), accompanied

by seminal plasma produced by the male reproductive system (Aire, 2014). The head contains a small acrosome (approximately 2.5 µm long and 0.5 µm wide in chickens) and the nucleus (6x0.5 µm in chickens). The number of chromosomes varies among species; for example, chicken spermatozoa have 41 chromosomes, while turkey spermatozoa have 39. Unlike mammals, where the heterogametic sex is male (XY), in birds it is female (ZW). Consequently, avian spermatozoa carry two Z chromosomes (McQueen and Clinton, 2009). The acrosome contains the acrosomal vesicle, which contains various hydrolytic enzymes such as acrosin, involved in digesting the inner perivitelline layer (IPVL) surrounding the oocyte, and the perforator, a rigid actin-based structure of unknown function in birds. The nucleus, surrounded by its double nuclear membrane, is highly condensed and serves to protect the genome during its long journey through the oviduct before fusing with the oocyte. In Passeriformes, spermatozoa have a helical coating surrounding the head and an intermediate portion containing proximal and distal centrioles, along with a highly variable number of mitochondria (approximately 30 in chickens and 150 in Japanese quails) that may extend along the flagellum depending on the species. This intermediate region acts as a "engine" for the energy metabolism of these gametes, which lack significant intracellular reserves but must be highly mobile to reach the sperm storage tubule (SST) and subsequently the oocyte.

The flagellum is the longest part of the cell, with an average length of 70-90 µm in chickens. It arises from the distal centriole and contains an axoneme with a central doublet and nine peripheral doublet microtubules, primarily composed of tubulin. In most birds (except *Passeriformes*), the axoneme is surrounded by a fibrous sheath for much of its length. During ejaculation, spermatozoa are diluted in seminal plasma, which is produced by various secretions and excretions from the male reproductive tract. This biological fluid has a neutral or slightly basic pH and is osmotically balanced due to its high content of amino acids such as glutamate and various proteins, the most abundant of which is serum albumin-like protein (Blesbois and Caffin, 1992). It also contains high amounts of cholesterol and phospholipids derived from numerous lipid and lipoprotein vesicles, including high-density lipoproteins and very high-density lipoproteins (Blesbois and Hermier, 1990; Douard et al., 2000). It also contains major inorganic ions (Na^+, K^+, Cl^-, $Ca2^+$, and Mg_2^+), carbohydrates like inositol and small amounts of glucose and glycerol, and hormones such as testosterone. Although seminal plasma stimulates sperm motility and is useful during ejaculation, it is not an ideal medium for long-term sperm preservation, despite the presence of high molecular weight fractions that support fertility. Consequently, it is eliminated before spermatozoa reach the female oviduct (Blesbois and De Reviers, 1992). Sperm concentration is generally quite high, ranging from about 30 million/mL to over 10 billion/mL in turkeys. This concentration can vary significantly depending on the species and breed.

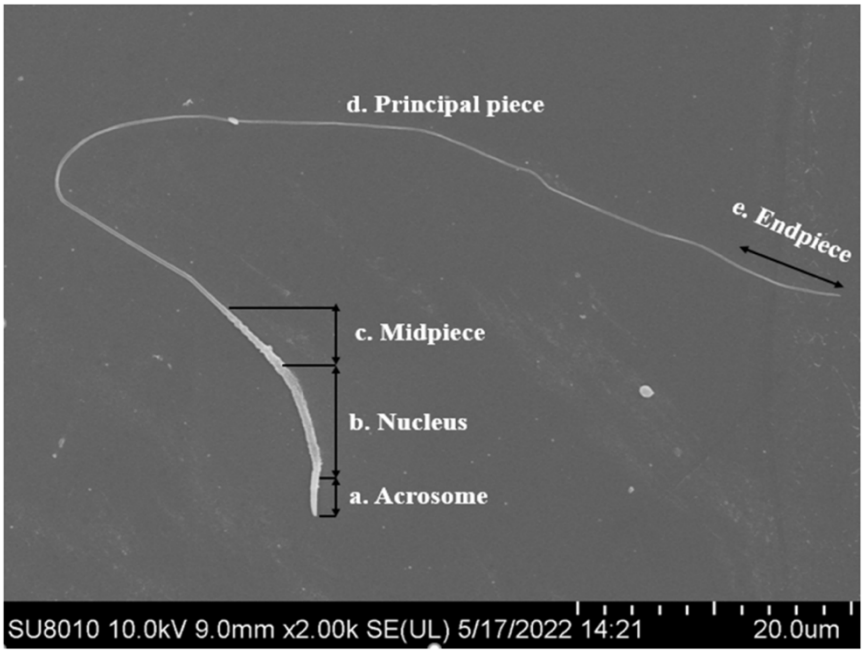


Figure 9 -The structure of Chicken sperm (scanning electron microscope, Hitachi su8010, Japan. scale bar = 20 um at 10,000× magnification). *Zong, Y. et al., 2023*

Part. 4

Safeguarding the animal biodiversity: LIFE Nat.Sal.Mo project and TuBAvI-2

My PhD activities are encompassed by two funded projects: the European Nat.Sal.Mo project and the national TuBAvI PSRN project. Hence, in this section it is imperative to delineate the objectives and outcomes of both project aiming at safeguard the animal biodiversity. These projects have a common aim, which is to preserve and valorize the local poultry breeds (TuBAvI projects), and native Mediterranean trout populations (Nat.Sal.Mo project), through the combined use of *in situ* and *ex situ* conservation strategies.

7. Recovery of S. Macrostigma: Application of Innovative Techniques and Participatory Governance Tools in rivers of Molise - LIFE Nat.Sal.Mo

In particular, in Italy, native populations of Mediterranean trout are rapidly declining, putting biodiversity at risk if appropriate conservation and protection measures are not taken. This decline is primarily caused by a series of anthropogenic activities. Numerous conservation initiatives have been implemented to tackle the urgent need for restoring genetic diversity within Mediterranean trout populations in Italy, as highlighted by studies (Sabatini et al., 2018; Lorenzoni et al., 2019; Splendiani et al., 2019; Rossi et al., 2022). These efforts were extended to the Molise region (Rusco et al., 2019; Rusco et al., 2020; Di Iorio et al., 2023), propelled notably by the European project LIFE Nat.Sal.Mo., which commenced in 2018 and concluded in 2023. The main aim of the LIFE Nat.Sal.Mo project was to ensure the recovery and conservation of the native Mediterranean trout in the river basins of Biferno and Volturno in the Molise region. This species, endemic to the Mediterranean area, is classified in Annex II of the Habitat Directive under the scientific name *Salmo macrostigma* and is currently reported as *Salmo cettii* in the Italian and European Red Lists and in the Member States conservation status assessments of the "habitat types and species of Community interest" (Art. 17). Recent studies indicate that Italian Mediterranean trout belong to a separate taxon called *S. ghigii*, limiting the name *S. cettii* to Sicilian trout (Lorenzoni et al., 2019; D'Agaro et al., 2022; Polgar et al., 2022). Although we agree with the recent observations, we still use the name *S. cettii* in the present doctoral thesis because Mediterranean brown trout populations are still protected by the Habitat Directive and subsequent updates on their conservation status. The Mediterranean trout is a freshwater fish of significant economic importance in terms of fisheries management and conservation biology. It has a wide distribution across Eurasia and North Africa, exhibiting substantial morphological, ecological, and genetic diversity among diverse populations (Larios-López et al., 2015).

The Mediterranean trout is a species that prefers clear waters and moderate currents with rocky and gravelly bottoms, but it can adapt to various environmental conditions. The reproductive season of the Mediterranean trout typically extends from late autumn to early spring, although this can vary depending on the population and environmental context. The intraspecific biological diversity of the Mediterranean trout is remarkable, primarily due to genetic differences among different populations. This genetic diversity is often accompanied by marked phenotypic variations. Furthermore, the significant variability in morphology and behavior among Mediterranean trout populations results from specific adaptations and plasticity influenced by local environmental conditions (Esposito et al., 2022). The native trout population in the project area exhibits a significant tendency for trophic and reproductive migrations, as confirmed by the results of Nat.Sal.Mo. The project area included the Volturno and Biferno rivers and their tributaries in the Molise region. The genetic analysis showed a different genetic introgression among sampling stations and between the two basins. In addition, by crossing the genetic analyses with the phenotypic characteristics, a reference phenotype of Molise native trout popolation (Figure 10) was identified that is characterised by 1) a blue-black preopercular mark (or halo); 2) frequent presence of 4 darker bands along the side that create a zebra-like pattern; 3) anterior margin of the anal and dorsal fin without a marked black and white band; 4) black or red spotting (frequently mixed) depending on environmental factors and not influenced by the introgression degree; 5) parr marks (when present, i.e. in the absence of vertical dark bands) always on more than one row and in a number greater than 9; 6) main livery pattern characterized by irregularly shaped spots and absence of oval spots surrounded by a rounded halo; 7) adipose fin without spots.

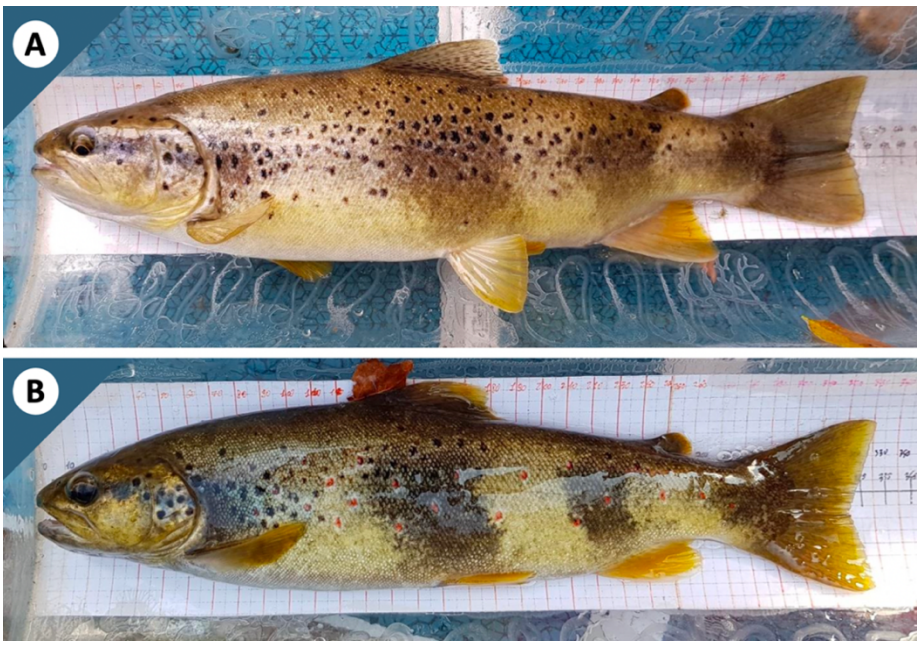


Figure 10 - Color patterns typically found in the main course of the Biferno (a) and Volturrno (b) rivers

The specific goals of the project were:

1) to recover the genetic integrity of the native populations of *S.macrostigma* threatened by introgressive hybridization. The reduction of the genetic introgression of the native trout populations in the project area occurred by:a) the selective access of native trout to spawning grounds by intercepting upstream migration with fixed traps and selecting wild breeders through genetic and morphological analysis; b) using semen from pure wild breeders for cryopreservation to use for artificial reproduction in combination with fertilization schemes to increase the genetic variability of offspring;

2) to protect and restore habitat functionalities, such as fluvial connectivity and quality of spawning grounds. The fluvial connectivity was implemented by construction of fish passes and the improvement of quality of spawning grawns occurred by removing waste from them;

3) to promote dispersion of native trout inside the project area with artificial reproduction and restocking of suitable areas using eyed eggs restocked by "artificial nesting". Two facilities for incubation were installed at Volturno basin and Biferno basin.

4) to update fishing regulations to support wild native trout populations;

5) to generate a positive social and economic impact;

6) to adopt participatory governance tools (i.e. River Contracts) to ensure future sustainability;

7) to assess replicability of methods developed in LIFE Nat.Sal.Mo. inside the Romanian territory and generate at least two replicability assessments in two Member States targeting at least one species other than the *S. macrostigma*.

The main threats to the *Mediterranean trout* in the project area are:

1. *Genetic Introgression:* The introduction of zootechnical strains into the Biferno and Volturno river basins, either for recreational purposes or due to escapes from zootechnical breeding, has caused genetic introgression. This has led to a decrease in the initial genetic diversity and ecological specializations that differentiate salmonid species. Introgressive hybridisation with non-native trout species poses a considerable risk to the survival of native Mediterranean trout population.

2. *Loss of Reproductive Habitat:* The loss of reproductive habitats is due to the presence of barriers in the tributary rivers of the main watercourses, preventing native trout from migrating to potential spawning sites. It is also due to the presence of pollution and waste that has altered some of the breeding areas. For example, the springs of the Biferno River (Bojano area) are frequented by many wild spawners during the egg-laying period but are heavily compromised by the presence of waste, including tires and appliances.

3. *Poorly Regulated Fishing:* Poorly regulated fishing represents another threat as it fails to effectively protect the species during the reproductive period through adequate fishing regulations.

In response to studies on biological characteristics, demographic parameters, and migratory patterns, fishing regulations have been developed in collaboration with local communities. These regulations include a ban on the introduction of non-native trout to preserve native populations. These threats pose significant challenges to the conservation of native Mediterranean trout in the Biferno and Volturno river basins. The innovative techniques employed in the NatSal.Mo project have proven instrumental in overcoming these challenges, resulting in better-than-expected outcomes. A significant reduction of genetic introgression in native trout populations in the project area was achieved through two innovative methodologies.

7.1 Selective access to spawning ground by fixed traps

This methodology consisted in an unusual system for the restoration of genetic integrity of native population inhabiting the Mediterranean rivers by installation of fixed traps. Selective access to spawning grounds for native trout was achieved by intercepting their upstream migration with fixed traps. Wild breeders were selected based on genetic and morphological analyses. This technique is widely used and tested in the exploitation and the management of other salmonid species characterized by spawning migration (eg. the north-America salmon hatcheries), to select wild and hatcheries-derived spawners. In the case of the Nat.Sal.Mo. project, the upstream traps were employed to compensate for the impossibility of carrying out traditional eradication techniques in watercourses with high flow rates, such as the main sections of the Volturno and Biferno rivers.

The action allowed us to: i) select the breeders with free access to the main natural spawning beds; ii) select the native females used for artificial reproduction; select the males for the starting process of the semen collection and its freezing; remove non-native individuals from the main spawning sites. In this regard, in total, 446 individuals were captured with traps, 246 native trout reached the spawning grounds, and 189 non-native trout were eliminated.

7.2 Semen cryobank

The establishment of the first sperm cryobank for populations of native Mediterranean trout is a priceless tool for the protection and conservation of this species' biodiversity. One of the main objectives of the LIFE Nat.Sal.Mo. project was to ensure the genetic integrity and biodiversity of the native Mediterranean brown trout population inhabiting the rivers of Molise. This was achieved by using frozen wild breeder semen associated with appropriate fertilization schemes to maximize genetic diversity in the progeny and maintain high fitness within self-sustaining populations. It is important to note that the level of introgression and inbreeding should be kept as low as possible to prevent detrimental effects on fitness-related traits, which could jeopardize the survival of the populations (Fernández et al., 2005). The frozen semen allowed us to avoid transferring wild fish into an artificial environment, that frequently causes significant loss by stress or domestication. In

Nat.Sal.Mo the semen stripping from native male to be frozen as well as the fertilization with cryopreserved semen was performed on the riverbed. During the project timeframe approximately 2,090 semen doses, from161 native breeders were stored inside the cryobank representing a very important “tank” of genetic variability, as it includes a large number of native donors captured in the Molise rivers during the spawning seasons. In this regard, 305,000 eggs from 88 native females were fertilized with 346 semen doses (from 161 donors) for a total of 346 unique male × female crosses to increase the genetical variability (fertilization schemes) (Table 7). Thus, the establishment of the first European cryobank for Mediterranean brown trout semen played a strategic role in conserving the existing genomic diversity of the native population, opening new opportunities to support hatchery management practices. The implementation of a native bloodstock park (live gene bank) is expected shortly in order to restock the waterways of our Molise region (south of Italy) with only autochthonous materials. However, the impoverishment of genetic variability and the loss of “rusticity” are among the main negative effects caused by the broodstock breeding in captivity that are transmitted and amplified to future generations. Breeding in captivity could lead to a dilution of “wild” genetic characters, in the course of generations dangerously exposing the populations at risk of extinction. Cryopreservation methodologies have the potential to minimize the losses of fitness and genetic diversity in long-term live-gene banking applications. Sperm cryobanking has significant advantages compared to breeding in captivity including labour, costs, and security; as a large number (hundreds/thousands) of samples from different generations can be preserved in relatively small spaces, avoiding the threat of damage from disease or genetic drift over time. Since, the genes within individual frozen spermatozoa remain mostly unchanged, genetic variation is not lost from the sample and no directional changes in allele frequencies can occur.

This technique maintains the genetic variability in individual frozen sperm and prevents directional changes in allele frequencies (O'Reilly and Doyle, 2007).

Table 7- Results obtained during three spawning seasons (2019-20; 2020-21; 2021-22)

Semen doses	Native breeders	Fertilized eggs	Fertility rates	Eyed eggs
2,091	161	305,000	> 75%	230,000

In the context of the LIFE Nat.Sal.Mo. project, the collection of semen from native males and the subsequent cryopreservation and artificial reproduction procedures were conducted directly on the riverbanks. This approach avoided the need to keep wild fish in artificial environments, a practice often associated with high mortality. A significant advantage of using cryopreserved semen is its ease of transport over time and space (Bøe et al., 2021). Cryopreserved semen can be stored almost indefinitely without undergoing substantial changes in motility and cellular vitality, which means it

can be used for multiple generations. (Bøe et al., 2021). To ensure the success of cryobanks, it was essential to develop an effective semen cryopreservation protocol. This procedure is notoriously stressful for sperm, which can be damaged during the process, compromising their function and structure. Fish sperm has various subcellular components, including the head, cytosol, plasma membrane, midpiece, and flagellum, all susceptible to freezing-induced damage, leading to DNA alteration, motility loss, plasma membrane rupture, and reduced fertilization capacity (Bozkurt and Yavas, 2021). During the project period, several experiments were conducted to develop the ideal cryopreservation protocol for Mediterranean trout. These studies involved the selection of suitable basic extenders, dilution rates, cryoprotectants (CPAs) and their concentrations, as well as freezing and thawing rates (Iaffaldano et al., 2016a; Di Iorio et al., 2019; Rusco et al., 2019; Rusco et al., 2020; Giametta et al., 2021). These efforts led to the identification of an effective protocol for semen cryopreservation, with specific details described in Rusco et al., 2020. It was also demonstrated that the protocol successfully works for semen samples collected up to 6 hours before and transported on ice.

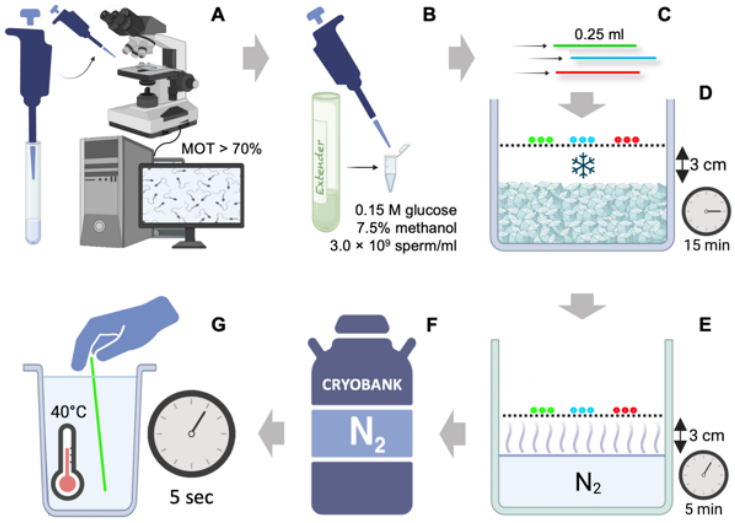


Figure 11 - A) Dilution of semen with an activation solution (1:300) for the evaluation of sperm quality using CASA system, to select samples with total motility > 70% B) Dilution of semen with a freezing extender (0.15 M glucose - 7.5% methanol) to achieve a final concentration of 3.0 x 10^9 spermatozoa/ml C) Loading of semen into 0.25 ml plastic straws D) Equilibration of the straws on a grid positioned 3 cm above ice for 15 minutes E) Freezing on nitrogen vapor by exposing the straws on a grid 3 cm above the liquid nitrogen surface for 5 minutes F) Storage of semen doses in liquid nitrogen (-196 °C) G) Prior to fertilization, the doses are thawed by immersing them in water at a temperature of 40 °C for 5 seconds using a portable thawing device.

To ensure proper management of the cryobanks and traceability of semen doses, all information about the doses was collected and continuously updated in a database. Each dose was identified by a serial number code that contains information about the donor. Liquid nitrogen containers are stored in a

climate-controlled environment at a controlled temperature of 5°C. Periodically, the storage containers are topped up with liquid nitrogen to ensure an adequate level.

Regarding the fertilization phase of the eggs with cryopreserved semen in the LIFE Nat.Sal.Mo. project, the eggs from each native female were divided into equal aliquots, each containing a maximum of about 1,000 eggs. These aliquots were fertilized with cryopreserved semen from different males according to a cross-fertilization scheme (Figure 12).

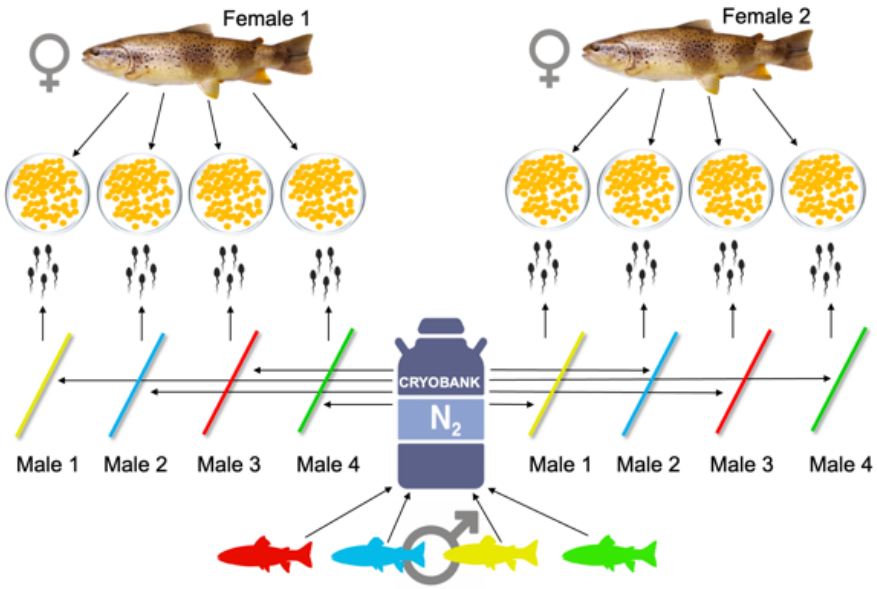


Figure 12 - The diagram shows the process of cross-fertilization using frozen semen. In the illustrated example, two females and four males are employed to generate eggs from eight distinct mating pairs (N males = 4; N females = 2; N pairs = 4 x 2 = 8).

The procedure involved thawing the cryopreserved semen, adding it to the eggs, and gentle mixing for 10 seconds. After 2 minutes, the eggs are rinsed with river water and transported to the incubators. The eggs are placed on a rack in flowing water with a temperature below 12°C and monitored until the embryonation stage. Dead and unfertilized eggs are removed twice a week, and the fertilization rate is calculated using the formula:

$$Fertilization\ rate\ (\%) = \frac{\text{Embryonated eggs}}{\text{Fertilized eggs}} \times 100$$

The cryopreservation technique of semen can result as useful in various situations:

- Artificial reproduction of wild breeders.
- Maintaining natural diversity levels in breeders.
- Overcoming asynchrony between males and females.

7.3 Artificial nesting

Another innovative technique used in Nat.Sal.Mo is the “artificial nesting” that assure a more successful approach because decreasing the non-adaptive selective pressure as much as possible by planting eyed eggs avoids the exposure of larvae and early life stages to an artificial environment. During the project 305,000 fertilized eggs were incubated at two hatcheries (one for each basin) and produced 230,000 eyed eggs (fertility rates averaging at 75%). The eyed eggs were planted using nesting techniques in suitable sites for the restocking program of native trout. The offspring are characterized by high genetic variability and consequently by a greater stability during seasons, protecting the natural reproductive ethology and the evolutionary potential, this gives a greater capacity of resilience to the native Mediterranean trout population. Briefly, this technique involves creating an artificial nest in the river gravel using a pipe, reaching a depth of approximately 20-30 cm. Subsequently, the eyed eggs are carefully placed inside the nest and covered with gravel and other natural materials. The nesting techniques emulates a natural redd.

8. Biodiversity conservation in Italian poultry breeds: TuBAvI-2 project

In recent decades, both in Italy and in the rest of the world, the poultry sector has suffered a significant loss of animal genetic resources and a progressive deterioration of many native genotypes due to the widespread use of high-performance commercial hybrids (Delany et al., 2004; Fulton et al., 2006). The advancement of intensive farming has put the survival of native chicken and turkey breeds at risk; the populations of these breeds are in fact maintained in extremely limited numbers and may be subject to inbreeding and loss of genetic diversity (Zanon and Sabbioni, 2001; Castillo et al., 2021). This has resulted in a loss of genetic diversity, making animals less adaptable to sudden environmental changes and more vulnerable to disease.

Safeguarding poultry biodiversity is a key objective in every developed country; however, little data is available on the extensive poultry genetic resources raised in Italy, and the urgent need for specific conservation programs for Italian poultry breeds is well recognized. In this context, the project "Conservation of biodiversity in Italian poultry breeds-TuBAvI" (www.pollitaliani.it) was developed from 2017 to 2020 with the financial support of the Ministry of Agricultural, Food and Forestry Policies (MIPAAF) and the European Fund Agriculture for Rural Development (EAFRD). The TuBAvI project aimed to promote and support the conservation of Italian poultry genetic resources by providing new data and tools for the implementation of a large and comprehensive national conservation program. Thanks to the TuBAvI project (Conservation of Biodiversity in Italian Poultry Breeds), seven Italian universities spread across the entire Italian territory including the University of Molise (UniMOL), the only university in the south of Italy collaborate to conserve avian biodiversity using an integrated and coordinated approach.

Information on the numerical consistency were collected, a phenotypic and genetic characterization of native breeds were carried out and the first national semen bank for the *Gallus gallus* and *Meleagris gallopavo species* was implemented. This project revealed that native breeds are mainly present in amateur exhibition breeding and that their population is critical, especially in Southern Italy.

The project included actions aimed at providing knowledge and new tools that can promote *in situ* and *ex situ in vitro* techniques for the conservation of biodiversity in the Italian poultry heritage.

The conservation of native chicken and turkey breeds is important not only to preserve their specific nutritional and nutraceutical qualities but also to enhance the resilience of populations to environmental stress and diseases. The loss of genetic variability has contributed to a decrease in the productive performance of these breeds.

Conservation techniques can be categorized into two main approaches: in situ and ex situ, with the latter further subdivided into in vivo and in vitro methodologies (FAO 2012; Leroy et al. 2019). In the in situ technique, living animals are maintained within the livestock production system in which

they originated, while the ex situ in vivo method involves housing them outside their native habitat. The *ex situ in vitro* technique entails cryopreserving genetic material in various forms such as haploid (semen and oocytes), diploid (embryos, somatic cells), or DNA sequences (FAO 2012; Mara et al. 2013). Cryopreserved materials are typically stored in specialized facilities known as gene banks or cryobanks, which are usually managed by universities, research centers, governments, or private companies (FAO 2012; Leroy et al. 2019). Despite the clear advantages of *in situ* conservation, which allows animals to evolve within their natural habitat, this method is often deemed too costly due to the need for dedicated infrastructure and careful management (Patterson and Silversides 2003; Prentice and Anzar 2011).

In avian species, the *ex situ in vitro* technique primarily relies on semen cryopreservation, as it is currently the most suitable reproductive technology for long-term storage of genetic resources (Long 2006; Blesbois 2011; Ehling et al. 2012). Cryopreservation of intact oocytes or embryos is not feasible due to the characteristics of megalecithal eggs (Long 2006; Blesbois et al. 2007, 2011; Ehling et al. 2012). Over the last decade, technologies for cryopreserving gonadic tissues and primordial germ cells have also been developed (Silversides et al. 2012; Liptoi et al. 2013; Nakamura et al. 2013, 2016; Sztan et al. 2017). However, these methods remain prohibitively expensive and highly invasive compared to semen cryopreservation.

Another project “Conservation of Biodiversity in Italian Poultry Breeds - TuBAvI-2," (PSRN 2014-2022 Measure 10.2 Biodiversity/Poultry sector - TuBAvI project n. 54250333926, with EAFRD support), was implemented, in order to strengthen the activities undertaken in the previous TuBAvI related to the recovery and preservation of avian genetic resources and at the same time other aspects were considered. Main goal is to increase the number of animals in endangered breed populations maintaining their genetic variability and promote their economic valorization within livestock farming systems focused on environmental sustainability. In TuBAvI-2, special attention is dedicated to enhancing resistance against adverse temperatures, climatic changes, and diseases in native poultry breeds, as these factors significantly impact their health and survival. Phenotypic characterization, which includes monitoring innate immunity parameters such as complement, IgY, and lysozyme levels, offers crucial insights into the birds' defense mechanisms against pathogens and contributes to understanding acquired immunity. These assessments aid in identifying breeds with greater resilience to diseases and climate variations, making them better suited for outdoor farming and the adoption of sustainable agricultural practices. However, maintaining a high level of genetic variability is equally essential for breed resilience, their capacity to adapt to climate change, and ensuring the safety of poultry products for consumers. Additionally, the productive characteristics of native poultry breeds are evaluated, encompassing growth rate, feed consumption, feed conversion ratio, mortality rate,

slaughter weight, and meat production. Parameters related to egg production, such as egg yield, egg weight, and egg size, are also assessed.

However, the University of Molise and the University of Milano, as partners of TuBAvI 2, persist in augmenting the semen doses stored in the cryobank for native breeds of chickens and turkeys.

REFERENCES

- Abd El-Emam, M. M., M. N. Ray, M. Ozono, and K. Kogure., 2023. Heat stress disrupts spermatogenesis via modulation of sperm-specific calcium channels in rats. J. Therm. Biol. 112:103465.
- Aegerter S., Jalabert B., 2004. Effects of post-ovulatory oocyte ageing and temperature on egg quality and on the occurrence of troploid fry in rainbow trout, *Oncorhynchus mykiss*. Aquaculture, 231, 59-71.
- Ahammad, M. U., C. Nishino, H. Tatemoto, N. Okura, Y. Kawamoto, S. Okamoto, and T. Nakada, 2011. Maturational changes in the survivability and fertility of fowl sperm during their passage through the male reproductive tract. Anim. Reprod. Sci. 128:129–136.
- Aire, T. A. (2014). Spermiogenesis in birds. Spermatogenesis 4:e959392.
- Alavi S.M.H., Cosson J., Bondarenko O., Linhart O., 2019. Sperm motility in fishes: (III). Diversity of regulatory signals from membrane to the axoneme. The-riogenology, 136, 143-165.
- Allan, J. D. & Flecker, A. S.,1993. Biodiversity conservation in running waters. BioScience 43, 32–43.
- Alonzo S.H., Stiver K.A., Marsh-Rollo S.E., 2016. Ovarian fluid allows directional cryptic female choice despite external fertilization. Nature Communications, 7, 12452.
- Amstislavsky, S. Y., & Trukshin, I. S., 2010. Cryobanking mammalian embryos: priorities and the optimal choice of reproductive technologies. Russian journal of developmental biology, 41, 13-23.
- ANCI – AIA- Registro delle Razze Avicola Autoctone https://www.anci-aia.it/il-libro-genealogico-delle-razze-avicole-autoctone/.
- Arlidge, N.S. J.W. Bull, P.F.E. Addison, M.J. Burgass, D. Gianuca, T.M. Gorham, C. Jacob, N. Shumway, S.P. Sinclair, J.E.M. Watson, C. Wilcox, E.J. Milner-Gulland, 2018. A global mitigation hierarchy for nature conservation.
- Asano, A., and C. Priyadarshana. 2022. Membrane-mediated regulation of sperm fertilization potential in poultry. J. Poult. Sci. 59:114–120.
- Ashizawa, K., and H. Nishiyama. 1978. Effects of temperature on the vigour of motility, oxygen consumption and duration of motility of fowl spermatozoa under aerobic conditions. Japanese Poult. Sci. 15:264–266.
- Avramova, Z., Uschewa, A., Stephanova, E., & Tsanev, R. (1983). Trout sperm chromatin. I. Biochemical and immunological study of the protein composition. European journal of cell biology, 31(1), 137-142.

- Beck, J. C., Fulcher, K. D., Beck, C. F., & Cloud, J. G. (1992). Sperm surface antigen required for fertility: identification on spermatozoa of rainbow trout by use of monoclonal antibodies. Transactions of the American Fisheries Society, 121(3), 333-339.
- Beirão J., Purchase C.F., Wringe B.F., Fleming I.A., 2015. Inter-population ovarian fluid variation differentially modulates sperm motility in Atlantic cod Gadus morhua. Journal of Fish Biology, 87, 54-68.
- Bermejo Delgado J. V., Martínez Martínez, M. A., Rodríguez Galván, G., Stemmer, A., Navas González, F. J., & Camacho Vallejo, M. E., 2019. Organization and management of conservation programs and research in domestic animal genetic resources. Diversity, 11(12), 235.
- Bernal, D. B., A. Behnamifar B, C. Álvarez-Rodríguez, A. Toledano-Díaz, C. Castanõ, R. Velázquez, M. G. Gil, A. Gutiérrez-Adán, H. Woelders, E. Blesbois, J. Santiago-Moreno, B. Bernal, and M. Yeste. 2022. Transit along the vas deferens results in a high percentage of filiform spermatozoa with compacted chromatin in the rooster (Gallus domesticus). Reprod. Fertil. Dev. 34:699–712.
- Berois N., Bollatto C., Brauer M., Barros C., 2004. Gametogenesis, histological gonadal cycle and in vitro fertilization in the whitemouth croaker (Micropogonias furnieri, Desmarest, 1823). Journal Applied Ichthyology, 20, 169-175.
- Berois N., Bollatto C., Brauer M., Barros C., 2004. Gametogenesis, histological gonadal cycle and in vitro fertilization in the whitemouth croaker (*Micropogonias furnieri*, Desmarest, 1823). Journal Applied Ichthyology, 20, 169-175.
- Berois, N., Arezo, M. J., & Papa, N. G.,2011. Gamete interactions in teleost fish: the egg envelope. Basic studies and perspectives as environmental biomonitor. Biological research, 44(2), 119-124.
- Billard, R. (1983). Effects of coelomic and seminal fluids and various saline diluents on the fertilizing ability of spermatozoa in the rainbow trout, Salmo gairdneri. Reproduction, 68(1), 77-84.
- Blesbois, E., 2011. Freezing avian semen. *Avian biology research*, *4*(2), 52-58.
- Bohrer, B. M. (2017). Nutrient density and nutritional value of meat products and non-meat foods high in protein. *Trends in Food Science & Technology*, *65*, 103-112.
- Bolton, R. L., A. Mooney, M. T. Pettit, A. E. Bolton, L. Morgan, G. J. Drake, R. Appeltant, S. L. Walker, J. D. Gillis, and C. Hvilsom. 2022. Resurrecting biodiversity: advanced assisted reproductive technologies and biobanking. Reprod. Fertil. 3:R121–R146.

- Borziak, K., A. Álvarez-Fernández, T. L. Karr, T. Pizzari, and S. Dorus. 2016. The Seminal fluid proteome of the polyandrous Red junglefowl offers insights into the molecular basis of fertility, reproductive ageing and domestication. Sci. Rep. 6:35864.
- Brummett A., Dumont J., 1979. Initial stages of sperm penetration into the egg of Fundulus heteroclitus. Journal of Experimental Zoology, 210, 417-434.
- Butts I.A.E., Johnson K., Wilson C.C., Pitcher T.E., 2012. Ovarian fluid enhances sperm velocity based on relatedness in lake trout, Salvelinus namaycush. Theri-ogenology, 78, 2105-2109.
- Butts I.A.E., Prokopchuk G., Kašpar V.,Cosson J., Pitcher T.E., 2017. Ovarian fluid impacts flagellar beating and biomechanical metrics of sperm between alternative reproductive tactics. Journal of Experimental Biology, 220, 2210-2217.
- Campbell, K. H. S., J. McWhir, W. A. Ritchie, and I. Wilmut. 1996. Sheep cloned by nuclear transfer from a cultured cell line. Nature 380:64–66.
- Castellini, C. Organic poultry production system and meat characteristics Proc XVII European Symposium on the Quality of Poultry Meat. Doorwerth 23/05/2005, 215.
- Castellini, C.; Berri, C.; Le Bihan-Duval, E.; Martino, G. Qualitative attributes and consumer perception of organic and free-range poultry meat. Worlds Poul. Sci. J. 2008, 65, 120-135.
- Castillo, A., Gariglio, M., Franzoni, A., Soglia, D., Sartore, S., Buccioni, A., ... & Schiavone, A. (2021). Overview of native chicken breeds in Italy: conservation status and rearing systems in use. Animals, 11(2), 490.
- Cendron F, Perini F, Mastrangelo S, Tolone M, Criscione A, Bordonaro S, Iaffaldano N, Castellini C, Marzoni M, Buccioni A, et al., 2020 Genome-Wide SNP Analysis Reveals the Population Structure and the Conservation Status of 23 Italian Chicken Breeds. *Animals*.; 10(8):1441.
- Chavatte-Palmer, P., R. Lee, M. Bertolini, H. Jammes, M. Schmidt, and H. Callesen. 2013. Pregnancy and neonatal care of SCNT animals. Princ. Cloning:107–216.
- Chen, L., Yin, L., Zhou, Q., Peng, P., Du, Y., Liu, L., ... & Cao, Y., 2019. Indagine epidemiologica sulle infezioni da adenovirus aviario nel pollame in Cina nel periodo 2015-2018. Ricerca veterinaria BMC , 15 , 1-7.
- Comizzoli, P., 2015. Biobanking efforts and new advances in male fertility preservation for rare and endangered species. Asian journal of andrology, 17(4), 640-645.
- Comizzoli, P., and D. E. Wildt., 2013. Mammalian fertility preservation through cryobiology: value of classical comparative studies and the need for new preservation options. Reprod. Fertil. Dev. 26:91–98.

- Comizzoli, P., Songsasen, N., & Wildt, D. E., 2010. Protecting and extending fertility for females of wild and endangered mammals. Oncofertility: Ethical, Legal, Social, and Medical Perspectives, 87-100.
- Commission on Genetic Resources for Food and Agriculture. Food and Agriculture Organization of the United Nations, Rome, Italy, 2007.
- Cosson J., 2010. Frenetic activation of fish spermatozoa flagella entails short-term motility, portending their precocious decadence. Journal of Fish Biology, 76, 240–279.
- Cosson J., 2019. Fish Sperm Physiology: Structure, Factors Regulating Motility, and Motility Evaluation. Recent Development. Journal of Fish Biology, 11-33.
- Costanza, R., R. de Groot, P. Sutton, S. van der Ploeg, S.J. Anderson, I. Kubiszewski, S. Farber, and R.K. Turner. 2014. Changes in the global value of ecosystem services. Global Environmental Change 26: 152–158.
- Dai, Y., L. Wang, H. Wang, Y. Liu, N. Li, Q. Lyu, D. L. Keefe, D. F. Albertini, and L. Liu. 2006. Fate of centrosomes following somatic cell nuclear transfer (SCNT) in bovine oocytes. Reproduction 131:1051–1061.
- Dal Bosco, A., Mattioli, S., Cartoni Mancinelli, A., Cotozzolo, E., & Castellini, C.,2021. Extensive Rearing Systems in Poultry Production: The Right Chicken for the Right Farming System. A Review of Twenty Years of Scientific Research in Perugia University, Italy. Animals
- Danchin-Burge, C., Bibe, B., & Planchenault, D.,2002. The French national cryobank: creation of a cryogenic collection for domestic animal species. Institut de l'élevage, 1-4.
- Davoli, R. 2011 "Biodiversità: un patrimonio da conservare privilegiando la qualità." La salvaguardia della biodiversita animale. Iniziative generali ed azioni intraprese in Italia a tutela delle razze minacciate. Brescia: Fondazione iniziative zooprofilattiche e zootecniche:1-3.
- Delgado, Christopher L. 2003 "Rising consumption of meat and milk in developing countries has created a new food revolution." The Journal of nutrition 133.11 (2003): 3907S-3910S.
- Deviche, P., L. L. Hurley, and H. B. Fokidis., 2011. Avian Testicular Structure, Function, and Regulation (D O, K Norris, and H Lopez, Eds.). Academic Press.
- Díaz, S., and Y. Malhi. 2022. Biodiversity: concepts, patterns, trends, and perspectives. Annu. Rev. Environ. Resour. 47:31–63.
- Dietrich G.J., Wojtczak M., Stowińska M., Dobosz S., Kuźmiński H., Ciereszko A., 2007. Broken eggs decrease pH of rainbow trout ovarian fluid. Aquaculture, 273, 748-751.
- Diogo P., Soares F., Dinis M.T., Cabrita E., 2010. The influence of ovarian fluid on Solea senegadensis sperm motility. Journal of Applied Ichthyology, 26, 690-695.

- Dudgeon, D., Arthington, A., Gessner, M. O., Kawabata, Z., Knowler, D., Lévêque, C., Naiman, R. J., Prieur-Richard, A., Soto, D., Stiassny, M. L. J., & Sullivan, C. A. ,2006. *Freshwater biodiversity: importance, threats, status and conservation challenges*. Biological Reviews/Biological Reviews of the Cambridge Philosophical Society.
- Dumont J., Brummett A., 1980. The vitelline envelope, chorion and micropyle of *Fundulus heteroclitus* eggs. Gamete Research, 3, 25-44.
- Dzyuba B.B., Van Look K.J., Kholodnyy V.S., Satake N, Cheung S., Holt W.V., 2008. Variable sperm size and motility activation in the pipefish, Syngnathus abas-ter; adaptations to paternal care or environmental plasticity?. Reproduction, Fertil-ity, Development, 20, 474-482.
- Estermann, M. A., A. T. Major, and C. A. Smith. 2021. Genetic Regulation of Avian Testis Development. Genes (Basel). 12:1459.
- Evans J.P., Sherman C.D.H., 2013. Sexual selection and the evolution of egg-sperm interactions in broadcast-spawning invertebrates. Biological Bulletin, 224, 166-183.
- FAO, 2000. World Watch List for Domestic Animal Diversity, third ed. FAO, Rome, Italy, 2000.
- FAO, 2007. The State of the World's Animal Genetic Resources for Food and Agriculture. Commission on Genetic Resources for Food and Agriculture. Food and Agriculture Organization of the United Nations, Rome, Italy, 2007.
- FAO, 2010. Breeding strategies for sustainable management of animal genetic resources. FAO Animal Production and Health Guidelines No. 3. Rome, Italy, 2010.
- FAO, 2011. The State of the World's Land and Water Resources for Food and Agriculture (SOLAW) – Managing Systems at Risk. Rome and London: Food and Agriculture Organization of the United Nations and Earthscan.
- FAO, 2012 Report *of a Consultation on the Definition of Breed Categories.* Commission on Genetic Resources for Food and Agriculture; Rome, Italy: 2012.
- FAO, 2012. Cryoconservation of animal genetic resources. FAO Animal Production and Health Guidelines No. 12. Rome, Italy, 2012.
- FAO, 2015. World Fertilizer Trends and Outlooks to 2018. Rome: Food and Agriculture Organization of the United Nations.
- FAO, 2020. Innovations in cryoconservation of animal genetic resources Practical guide
- FAO,2022. Intergovernmental technical working group on animal genetic resources for food and agriculturethe State of the World's Animal Genetic Resources for Food and Agriculture; Status and trends of animal genetic resources Rome, Italy, 2020.
- FAO, 2023 "Food Safety and Control Newsletter, Vol. 02, No. 2, July 2023."

- Floros, J. D., Newsome, R., Fisher, W., Barbosa-Cánovas, G. V., Chen, H., Dunne, C. P., ... & Ziegler, G. R. (2010). Feeding the world today and tomorrow: the importance of food science and technology: an IFT scientific review. *Comprehensive Reviews in Food Science and Food Safety*, *9*(5), 572-599.
- Fruciano, C., A. M. Pappalardo, C. Tigano, and V. Ferrito. 2014. Phylogeographical relationships of Sicilian brown trout and the effects of genetic introgression on morphospace occupation. Biol. J. Linn. Soc. 112: 387–398.
- Fujihara, N. 1992. Accessory reproductive fluids and organs in male domestic birds. World's Poult. Sci. J. 48:39–56.
- Galarza, D. A., G. Landi, E. Mej.a, J. X. Samaniego, S. M.ndez, M. E. Soria, J. Taboada, M. J. Sanchez-Calabuig, C. Casta.o, and J. Santiago-Moreno. 2021. Cryopreservation of dog epididymal spermatozoa by conventional freezing or ultra-rapid freezing with nonpermeable cryoprotectant. Cryobiology 103:15–21.
- Gandini, G., & Oldenbroek, K. (2007). Strategies for moving from conservation to utilisation. In Utilisation and conservation of farm animal genetic resources (pp. 29-54). Wageningen Academic.
- Gasparini C., Andreatta G., Pilastro A., 2012. Ovarian fluid of receptive females enhances sperm velocity. Naturwissenschaften, 99, 417–420.
- Gasparini C., Evans J.P., 2013. Ovarian fluid mediates the temporal decline in sperm viability in fish with sperm storage. PLoS One, 8 (5), 64431.
- Gasparini, C., & Pilastro, A. (2011). Cryptic female preference for genetically unrelated males is mediated by ovarian fluid in the guppy. Proceedings of the Royal Society B: Biological Sciences, 278(1717), 2495-2501.
- Geßner C., Johnson S.L., Fisher P., Clarke S., Rutherford K., Symonds J., et al., 2017. Male-female relatedness at specific SNP-linkage groups influences cryptic female choice in Chinook salmon (*Oncorhynchus tshawytscha*). Proceedings of the Royal Society B, Biological Sciences 284 (1859), 20170853.
- Gibbons, M.; Goodsell, J.G.; Castagna, M.; Luz, R. Chemical stimulation of spawning by serotonin in the ocean quahog Artica islandica (Linné). J. Shellfish Res. 1983, 3, 203–205.
- Gouk, S.S.; Loh, Y.F.; Kumar, S.D.; Watson, P.F.; Kuleshova, L.L. Cryopreservation of mouse testicular tissue: prospect for harvesting spermatogonial stem cells for fertility preservation. Fertil. Steril. 2011, 95, 2399–403.

- Gozlan, R.E., B.K. Karimov, E. Zadereev, D. Kuznetsova, and S. Brucet. 2019. Status, trends, and future dynamics of freshwater ecosystems in Europe and Central Asia. Inland Waters 9: 78–94.
- Hafez, Hafez M., and Youssef A. Attia. "Challenges to the poultry industry: current perspectives and strategic future after the COVID-19 outbreak." Frontiers in veterinary science 7 (2020): 516.
- Hammond, K., & Galal, S. (2000). Developing breeding strategies for lower input animal production environments. An Introduction. Developing breeding Strategies for Lower Input Animal Production Environments, 13.
- Hart N., Donovan M., 1983. Fine structure of the chorion and site of sperm entry in the egg of Brachydanio. Journal of Experimental Zoology, 227, 277-296.
- Haseeb, A., X. Bai, W. A. Vistro, I. Tarique, H. Chen, P. Yang, N. S. Gandahi, A. Iqbal, Y. Huang, and Q. Chen. 2019. Characterization of in vivo autophagy during avian spermatogenesis. Poult. Sci. 98:5089–5099.
- Hassink, J., Dijk, M. V., & Bogers, R. J. (2005). Farming for health. Proceedings of the Frontis Workshop on farming for health, Wageningen, The Netherlands, 16-19 March, 2005. In Farming for health. Proceedings of the Frontis Workshop on farming for health, Wageningen, The Netherlands, 16-19 March, 2005. Wageningen Universiteit (Wageningen University).
- Herrid, M.; Vignarajan, S.; Davey, R.; Dobrinski, I.; Hill, J.R. Successful transplantation of bovine testicular cells to heterologous recipients. Reproduction 2006, 132, 617–24.
- Hoshino, Y., and K. Saeki. 2010. Animal cloning by nuclear transfer using somatic cells recovered from organs frozen without cryoprotectant. J. Mamm. Ova Res. 27:93–100.
- Howarth Jr, B., 1971. Transport of spermatozoa in the reproductive tract of turkey hens. *Poultry science*, *50*(1), 84-89.
- Howarth, B. 1971. An examination for sperm capacitation in the fowl. Biol. Reprod. 3:338–341.
- Iaffaldano, N., Di Iorio, M., Manchisi, A., Gibertoni, P. P., & Esposito, S. (2016a). Semen quality of threatened native population of Mediterranean brown trout (Salmo cettii, Rafinesque 1810) in the Biferno River (Molise Region-South Italy). *Turkish journal of fisheries and aquatic sciences*, *16*(2), 259-266.
- Iaffaldano, N.; Di Iorio, M.; Manchisi, A.; Esposito, S.; Gibertoni, P.P. Effective freezing rate for semen cryopreservation in endangered Mediterranean brown trout (Salmo trutta macrostigma) inhabiting the Biferno river (South Italy). Zygote 2016a, 24, 668-675.
- Inaba, K.; Dréanno, C.; Cosson, J.; et al. Control of flatfish sperm motility by CO2 and carbonic anhydrase. Cell. Motil. Cytoskeleton 2003, 55, 174–187.

- Inanan B.E., Öğretmen F., 2015. Determination of differences in the biochemical properties of sperm activating and non-activating ovarian fluids and their influ-ences on sperm motility in rainbow trout. Aquaculture, 448, 539-44.
- Iniguez, L. (2005). *Characterization of small ruminant breeds in West Asia and North Africa* (Vol. 1). Aleppo: ICARDA.
- IPBES. 2018. IPBES Guide on the production of assessments. Bonn, Germany: Secretariat of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services.
- Iqbal, A., J. Ping, S. Ali, G. Zhen, J. Z. Kang, P. Z. Yi, L. Huixian, and Z. Zhihui. 2021. Conservation of endangered species through somatic cell nuclear transfer (SCNT). Conserv. Genet. Resour. 13:349–357.
- IUCN. 2023. The IUCN Red List of Threatened Species. : https://www.iucnredlist.org.
- Jackson, R. B., Carpenter, S. R., Dahm, C. N., Mcknight, D. M., Naiman, R. J., Postel, S. L. & Running, S. W. (2001). Water in a changing world. Ecological Applications 11, 1027–1045.
- Johnson, S. L., Villarroel, M., Rosengrave, P., Carne, A., Kleffmann, T., Lokman, P. M., & Gemmell, N. J. (2014). Proteomic analysis of chinook salmon (*Oncorhynchus tshawytscha*) ovarian fluid. PloS one, 9(8), e104155.
- Kang, S.J.; Choi, J.W.; Kim, S.Y.; Park, K.J.; Kim, T.M.; Lee, Y.M.; Kim, H.; Lim, J.M.; Han, J.Y. Reproduction of wild birds via interspecies germ cell transplantation. Bio. Reprod. 2008, 79, 931–941.
- Kanuga M.K., Drew R.E., Wilson-Leedy J.G., Ingermann R.L., 2012. Subpopula-tion distribution of motile sperm relative to activation medium in steelhead (*Oncorhynchus mykiss*). Theriogenology, 77, 916-925.
- Kim, M.S.; Pinto, S.M.; Getnet, D.; Nirujogi, R.S.; Manda, S.S.; Chaerkady, R.; Madugundu, A.K. et al. A draft map of the human proteome. Nature 2014, 509(7502), 575–581.
- Kobayashi, T.; Takeuchi, Y.; Takeuchi, T.; Yoshizaki, G. Generation of viable fish from cryopreserved primordial germ cells. Mol. Reprod. Dev. 2007, 74, 207–13.
- Kottelat M, Freyhof J (2007) *Handbook of European freshwater fishes*. Publications Kottelat, Cornol, Switzerland.
- Labbé, C., & Maisse, G. (2001). Characteristics and freezing tolerance of brown trout spermatozoa according to rearing water salinity. Aquaculture, 201(3-4), 287-299
- Lahnsteiner F., Berger B., Weismann T., Patzner R. A., 1996. Motility of sperma-tozoa of Alburnus alburnus (Cyprinidae) and its relationship to seminal plasma composition and sperm metabolism. Fish Physiological Biochemistry 15, 167–179.

- Lahnsteiner F., Weismann T., Patzner R.A., 1999. Physiological and biochemical parameters for egg quality determination in lake trout, *Salmo trutta lacustris*. Fish Physiol Biochem, 20, 375-88.
- Lahnsteiner, F., Berger, B., Weismann, T., & Patzner, R. A.,1998. Determination of semen quality of the rainbow trout, Oncorhynchus mykiss, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. Aquaculture, 163(1-2), 163-181.
- Lahnsteiner, F., Mansour, N., & Weismann, T., 2002. The cryopreservation of spermatozoa of the burbot, Lota lota (Gadidae, Teleostei). Cryobiology, 45(3), 195-203
- Lahnsteiner, Franz, and Robert Patzner., 2008 "Sperm morphology and ultrastructure in fish." Fish Spermatology (eds.: SMH Alavi, J. Cosson, K. Coward, G. Rafiee. Alpha Science International Ltd. Oxford, UK. Alpha Science International Ltd., 1-61
- Lam T., Nagahama Y., Chan K., Hoar Ws., 1978. Overripe eggs and postovulatory corpora lutea in the threespine stickleback, *Gasterosteus aculeatus* L., form trachu-rus. Canadian Journal of Zoology, 56, 2029-2036.
- Lehnert S.J., Butts I.A.E., Flannery E.W., Peters K.M., Heath D.D., Pitcher T.E., 2017. Effects of ovarian fluid and genetic differences on sperm performance and fertilization success of alternative reproductive tactics in Chinook salmon. Journal of Evolutionary Biology, 30, 1236-1245.
- Leibo, S. P., and N. Songsasen. 2002. Cryopreservation of gametes and embryos of nondomestic species. Theriogenology 57:303–326.
- Lemoine, M., I. Grasseau, J. P. Brillard, and E. Blesbois. 2008. A reappraisal of the factors involved in in vitro initiation of the acrosome reaction in chicken spermatozoa. Reproduction 136:391–399.
- Leung, L.K.P.; Jamieson, B.G.M. Live preservation of fish gametes, In Fish Evolution and Systematics: Evidence from Spermatozoa, (ed. B.G.M. Jamieson), Cambridge University Pres., 991, pp. 245-95.
- Li, Y., Y. Sun, A. Ni, L. Shi, P. Wang, A. M. Isa, P. Ge, L. Jiang, J. Fan, H. Ma, G. Yang, and J. Chen. 2020. Seminal plasma proteome as an indicator of sperm dysfunction and low sperm motility in chickens. Mol. Cell. Proteomics 19:1035–1046.
- Liberti J., Baer B., Boomsma J.J., 2016. Queen reproductive tract secretions en-hance sperm motility in ants. Biology Letters, 12 (11), 20160722.
- Linhartova, P., Gazo, I., Shaliutina, A., & Hulak, M. (2013). The in vitro effect of duroquinone on functional competence, genomic integrity, and oxidative stress indices of sterlet (Acipenser ruthenus) spermatozoa. Toxicology in Vitro, 27(6), 1612-1619.

- Liptoi, K., Horvath, G., Gal, J., Varadi, E., & Barna, J., 2013. Preliminary results of the application of gonadal tissue transfer in various chicken breeds in the poultry gene conservation. *Animal reproduction science*, *141*(1-2), 86-89.
- Lobón-Cerviá, J., Esteve, M., Berrebi, P., Duchi, A., Lorenzoni, M., & Young, K. A. (2019). Trout and char of central and southern Europe and northern Africa. Trout and char of the world, 379-410.
- Long JA., 2006. Avian semen cryopreservation: what are the biological challenges? Poult Sci. 85(2):232–236.
- Lorenzoni, M., Carosi, A., Giovannotti, M., La Porta, G., Splendiani, A., & Barucchi, V. C. (2019). Ecology and conservation of the Mediterranean trout in the central Apennines (Italy). Journal of limnology, 78(1).
- Lundberg, J. G., Kottelat, M., Smith, G. R., Stiassny, M. L., & Gill, A. C. (2000). So many fishes, so little time: an overview of recent ichthyological discovery in continental waters. Annals of the Missouri Botanical Garden, 26-62.
- Lundberg, John G., et al. "So many fishes, so little time: an overview of recent ichthyological discovery in continental waters." Annals of the Missouri Botanical Garden (2000): 26-62.
- MA (2005) Millennium ecosystem assessment. Ecosystems and human well-being. World Resources Institute (Washington DC: Island Press)
- Malmqvist, B., & Rundle, S., 2002. Threats to the running water ecosystems of the world. Environmental conservation, 29(2), 134-153.
- Malmqvist, B., Monaghan, M., Morin, P. J. & O'Mullan, G.,2004. Biodiversity effects on ecosystem functioning: emerging issues and their experimental test in aquatic environments. Oikos 104, 423–431.
- Mara, L., Casu, S., Carta, A., & Dattena, M. (2013). Cryobanking of farm animal gametes and embryos as a means of conserving livestock genetics. *Animal Reproduction Science*, *138*(1-2), 25-38.
- Martínez-Fresneda, L., C. Castaño, P. Bóveda, D. Tesfaye, K. Schellander, J. Santiago-Moreno, and F. A. García-Vazquez., 2019. Epididymal and ejaculated sperm differ on their response to the cryopreservation and capacitation processes in mouflon (Ovis musimon). Sci. Rep. 9:15659.
- Meuwissen, T., 2007. Operation of conservation schemes. In Utilisation and conservation of farm animal genetic resources (pp. 167-193). Wageningen Academic.
- Morita, T.; Kumakura, N.; Morishima, K.; Mitsuboshi, T.; Ishida, M.; Hara, T.; Kudo, S., Miwa, M.; Ihara, S.; Higuchi, K.; Takeuchi, Y.; Yoshizaki, G. Production of donor-derived offspring by

allogenic transplantation of spermatogonia in the yellowtail (Seriola quinqueradiata). Biol. Reprod. 2012, 86, 1–11.

- Mottet, A., & Tempio, G. (2017). Global poultry production: current state and future outlook and challenges. World's poultry science journal, 73(2), 245-256.
- Moyle, Peter B., and Robert A. Leidy. "Loss of biodiversity in aquatic ecosystems: evidence from fish faunas." *Conservation biology: The theory and practice of nature conservation preservation and management*. Boston, MA: Springer US, 1992. 127-169.
- Munoz-Guerra, S., Azorin, F., Casas, M. T., Marcet, X., Maristany, M. A., Roca, J., & Subirana, J. A.,1982. Structural organization of sperm chromatin from the fish Carassius auratus. Experimental Cell Research, 137(1), 47-53.
- Naiman, R. J. & Turner, M. G., 2000. A future perspective on North America's freshwater ecosystems. Ecological Applications 10, 958–970.
- Neff B.D., Pitcher T.E., 2005, Genetic quality and sexual selection: an integrated framework for good genes and compatible genes. Molecular Ecology, 14, 19-38.
- Nelson, S. M. (Ed.). (2006). Handbook of gender in archaeology. Rowman Altamira.
- Nixon, B., K. A. Ewen, K. M. Krivanek, J. Clulow, G. Kidd, H. Ecroyd, and R. C. Jones. 2014. Post-testicular sperm maturation and identification of an epididymal protein in the Japanese quail (*Coturnix coturnix japonica*). Reproduction 147:265–277.
- Nynca J., Arnold G.J., Frōhlich T., Ciereszko A., 2015. Shotgun proteomics of rainbow trout ovarian fluid. Reproduction, Fertility and Development, 27, 504-12.
- Oback, B. 2008. Climbing Mount Efficiency--small steps, not giant leaps towards higher cloning success in farm animals. Reprod. Domest. Anim. 43:407–416.
- Ohta, Y., Sato, T., & Iguchi, T. (1993). Immunocytochemical localization of progesterone receptor in the reproductive tract of adult female rats. Biology of reproduction, 48(1), 205-213.
- Okutsu, T.; Suzuki, K.; Takeuchi, Y.; Yoshizaki, G. Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proc. Natl. Acad. Sci. USA 2006, 103, 2725–9.
- Ono, T.; Yokoi, R.; Aoyama, H. The transfer of male or female primordial germ cells of quail into chick embryonic gonads. Exp. Anim. 1996, 45, 347–352.
- Ormerod, S. J. (2003). Current issues with fish and fisheries: editor's overview and introduction. Journal of Applied Ecology, 40(2), 204-213.
- Ovaska, Ulla, and Katriina Soini. "Local breeds–Rural heritage or new market opportunities? Colliding views on the conservation and sustainable use of landraces." Sociologia Ruralis 57 (2017): 709-729.

- Padhi, A., M. M. Ghaly, and L. Ma. 2016. Testis-enriched heat shock protein A2 (HSPA2): Adaptive advantages of the birds with internal testes over the mammals with testicular descent. Sci. Rep. 6:18770.
- Papachristoforou, C.; Koumas, A.; Hadjipavlou, G. Adding value to local breeds with particular reference to sheep and goats. Anim. Genet. Resour. 2013, 53, 157–162.
- Patterson, D. L., and F. G. Silversides., 2003. "Farm animal genetic resource conservation–why and how." Why and how.
- Prentice, J. R., & Anzar, M.,2011. Cryopreservation of mammalian oocyte for conservation of animal genetics. *Veterinary medicine international*, *2011*.
- Rahel, F. J. (2002). Homogenization of freshwater faunas. Annual Review ofEcology and Systematics 33, 291–315.
- Rege, J. E. O., & Gibson, J. P. (2003). Animal genetic resources and economic development: issues in relation to economic valuation. *Ecological economics*, *45*(3), 319-330.
- Reid, A. J., Carlson, A. K., Creed, I. F., Eliason, E. J., Gell, P. A., Johnson, P. T., ... & Cooke, S. J. (2019). Emerging threats and persistent conservation challenges for freshwater biodiversity. Biological reviews, 94(3), 849-873.
- Reid, G. McG.; Contreras MacBeath, T.; Csatadi, K. Global challenges in freshwater fish conservation related to public aquariums and the aquarium industry. Int. Zoo Yearbook, 2013, 47(1), 6-45.
- Revenga, C., Campbell, I., Abell, R., De Villiers, P. & Bryer, M. (2005). Prospects for monitoring freshwater ecosystems towards the 2010 targets. PhilosophicalTransactions ofthe RoyalSociety B360, 397–413.
- Rischkowsky, B., & Pilling, D. (2007). The state of the world's animal genetic resources for food and agriculture. Food & Agriculture Org.
- Robeck, T. R., S. A. Gearhart, K. J. Steinman, E. Katsumata, J. D. Loureiro, and J. K. O'Brien. 2011. *In vitro* sperm characterization and development of a sperm cryopreservation method using directional solidification in the killer whale (*Orcinus orca*). Theriogenology 76:267–279.
- Roques, S., Chancerel, E., Boury, C., Pierre, M., & Acolas, M. L., 2019. From microsatellites to single nucleotide polymorphisms for the genetic monitoring of a critically endangered sturgeon. Ecology and evolution, 9(12), 7017-7029.
- Rosengrave P., Montgomerie R., Metcalf V.J., McBride K., Gemmell N.J., 2009b. Sperm traits in Chinook salmon depend upon activation medium: implications for studies of sperm competition in fishes. Canadian Journal Zoology, 87, 920-927.

- Rosengrave P., Taylor H., Montgomerie R., Metcalf V., McBride K., Gemmell Nj., 2009a. Chemical composition of seminal and ovarian fluids of chinook salmon and their effects on sperm motility traits. Comparative Biochemistry and Physiology A, 152, 123-129.
- Rosengrave, P., Montgomerie, R., & Gemmell, N., 2016. Cryptic female choice enhances fertilization success and embryo survival in chinook salmon. Proceedings of the Royal Society B: Biological Sciences, 283(1827), 20160001.
- Rossi, A. R., Talarico, L., Petrosino, G., Crescenzo, S., & Tancioni, L.,2022. Conservation genetics of Mediterranean brown trout in central Italy (Latium): A multi-marker approach. Water, 14(6), 937.
- Roufidou C., 2017. Reproductive physiology of the female three-spined stickle-back, Gasterosteus aculeatus. PhD thesis. Stockholm: Stockholm University, 65.
- Ruane, J. (2000). A framework for prioritizing domestic animal breeds for conservation purposes at the national level: a Norwegian case study. Conservation Biology, 14(5), 1385-1393.
- Ryan, J.P. Cryobank Management. In S.D. Fleming, A.C. Varghese eds. Organization and Management of IVF Units. New York, 2016.
- Santiago-Moreno, J., and E. Blesbois. 2020. Functional Aspects of Seminal Plasma in Bird Reproduction. Int. J. Mol. Sci. 21:5664.
- Santiago-Moreno, J., and E. Blesbois. 2022. Animal board invited review: Germplasm technologies for use with poultry. Animal 16:100475.
- Santiago-Moreno, J., B. Bernal, S. Pérez-Cerezales, C. Castaño, A. Toledano-Díaz, M. C. Esteso, A. Gutiérrez-Adán, A. López-Sebastián, M. G. Gil, H. Woelders, and E. Blesbois. 2019. Seminal plasma amino acid profile in different breeds of chicken: Role of seminal plasma on sperm cryoresistance. PLoS One 14:e0209910.
- Saperas, N., Lloris, D., & Chiva, M. (1993). Sporadic appearance of histones, histone-like proteins, and protamines in sperm chromatin of bony fish. Journal of Experimental Zoology, 265(5), 575-586.
- Satia B.P., Donaldson L.R., Smith L.S., Nightingale J.N., 1974. Composition of ovarian fluid and eggs of the university of Washington strain of rainbow trout (*Salmo gairdneri*). Journal of the Fisheries Research Board of Canada, 31, 1976-1979.
- Savvulidi, F., Ptacek, M., Savvulidi Vargova, K., & Stadnik, L. (2019). Manipulation of spermatogonial stem cells in livestock species. Journal of animal science and biotechnology, 10, 1-18.
- Schiewe, M. C., M. Bush, V. De Vos, J. L. Brown, E. Wildt, M. C. Schiewe, M. Bush, and V. De Vos. 1991. Semen characteristics, sperm freezing, and endocrine profiles in free-living wildebeest

(*Connochaetes taurinus)* and greater kudu (Tragelaphus strepsiceros). J. Zoo Wildl. Med. 22:58–72.

- Schöffmann, J., Sušnik, S., & Snoj, A. (2007). Phylogenetic origin of Salmo trutta L 1758 from Sicily, based on mitochondrial and nuclear DNA analyses. Hydrobiologia, 575, 51-55.
- Silversides, F. G., Purdy, P. H., & Blackburn, H. D., 2012. Comparative costs of programmes to conserve chicken genetic variation based on maintaining living populations or storing cryopreserved material. *British poultry science*, *53*(5), 599-607.
- Skarstein F., Folstad I., Liljedal S., Grahn M., 2005. MHC and fertilization success in the Arctic charr (*Salvelinus alpinus)*. Behavioral Ecology and Sociobiology, 57, 374-380.
- Słowińska, M., Ł. Paukszto, J. Paweł Jastrzębski, J. Bukowska, K. Kozłowski, J. Jankowski, and A. Ciereszko. 2020. Transcriptome analysis of turkey (Meleagris gallopavo) reproductive tract revealed key pathways regulating spermatogenesis and post-testicular sperm maturation. Poult. Sci. 99:6094–6118.
- Soini, K. and Y. de Haas, 2010. Trends in cattle diversity and cattle production in Europe: from popular to niche. Pp. 22–39 in S.J. Hiemstra, Y. Haas, A. Mäki-Tanila et al. eds, Local cattle breeds in Europe: development of policies and strategies for self-sustaining breeds (Wageningen: Wageningen Academic Publishers)
- Sonesson, A. K., Goddard, M. E., & Meuwissen, T. H. (2002). The use of frozen semen to minimize inbreeding in small populations. Genetics Research, 80(1), 27-30.
- Song, Y.; Silversides, F.G. Production of offspring from cryopreserved chicken testicular tissue. Poult. Sci. 2007b, 86, 1390–1396.
- Soule, M. A., Barange, M., and Hampton, I. 1995. Evidence of bias in estimates of target strength obtained with a split-beam echo-sounder. ICES Journal of Marine Science, 52: 139–144
- Soulé, M. E. (1985). What is conservation biology?. BioScience, 35(11), 727-734.
- Soulé, Michael E. "Conservation: tactics for a constant crisis." Science 253.5021., 1991: 744-750.
- Splendiani, A., Palmas, F., Sabatini, A., & Caputo Barucchi, V., 2019. The name of the trout: considerations on the taxonomic status of the Salmo trutta L., 1758 complex (Osteichthyes: Salmonidae) in Italy. *The European Zoological Journal*, *86*(1), 432-442.
- Splendiani, A., Ruggeri, P., Giovannotti, M., Pesaresi, S., Occhipinti, G., Fioravanti, T., ... & Caputo Barucchi, V., 2016. Alien brown trout invasion of the Italian peninsula: the role of geological, climate and anthropogenic factors. *Biological invasions*, *18*, 2029-2044.
- Sprando, R. L., and L. D. Russell., 1988. Spermiogenesis in the red-ear turtle (Pseudemys scripta) and the domestic fowl (Gallus domesticus): A study of cytoplasmic events including cell volume changes and cytoplasmic elimination. J. Morphol. 198:95–118.

- Stoops, M. A., J. B. Bond, H. L. Bateman, M. K. Campbell, G. P. Levens, T. R. Bowsher, S. T. Ferrell, W. F. Swanson, M. A. Stoops, J. B. Bond, H. L. Bateman, M. K. Campbell, G. P. Levens, T. R. Bowsher, S. T. Ferrell, and W. F. Swanson. 2007. Comparison of different sperm cryopreservation procedures on post-thaw quality and heterologous *in vitro* fertilization success in the ocelot (*Leopardus pardalis*). Reprod. Fertil. Dev. 19:685–694.
- Sztan N, Lazar B, Bodzsar N, Vegi B, Liptoi K, Pain B, Varkonyi EP., 2017. Successful chimera production in the Hungarian goose (*Anser anser domestica*) by intracardiac injection of blastodermal cells in 3-day-old embryos. Reprod Fert Develop. 29:2206–2216.
- Tacconi, L. Biodiversity and Ecological economics: participation, value and resource management. Earth scan publication. Ltd London, 2000.
- Talarico, L., et al. "Population structure, genetic diversity and demographic patterns unveil massive Mediterranean brown trout manipulations in a protected area of the northern Apennines (Italy)." The European Zoological Journal 90.1 (2023): 470-486.
- Thélie, A., S. Rehault-Godbert, J. C. Poirier, M. Govoroun, S. Fouchécourt, and E. Blesbois. 2019b. The seminal acrosin-inhibitor ClTI1/SPINK2 is a fertility-associated marker in the chicken. Mol. Reprod. Dev. 86:762–775.
- Tisdell, C. (2003). Socioeconomic causes of loss of animal genetic diversity: analysis and assessment. *Ecological Economics*, *45*(3), 365-376.
- Tougard, C., Justy, F., Guinand, B., Douzery, E. J., & Berrebi, P. (2018). Salmo macrostigma (Teleostei, Salmonidae): nothing more than a brown trout (S. trutta) lineage?. Journal of Fish Biology, 93(2), 302-310.
- Tubbs, C., & Thomas, P. (2008). Functional characteristics of membrane progestin receptor alpha (mPRα) subtypes: a review with new data showing mPRα expression in seatrout sperm and its association with sperm motility. Steroids, 73(9-10), 935-941.
- Ugur, M. R., A. Saber Abdelrahman, H. C. Evans, A. A. Gilmore, M. Hitit, R. I. Arifiantini, B. Purwantara, A. Kaya, and E. Memili. 2019. Advances in cryopreservation of bull sperm. Front. Vet. Sci. 6:268.
- Urbach D., Folstad I., Rudolfsen G., 2005. Effects of ovarian fluid on sperm veloc-ity in Arctic charr *(Salvelinus alpinus).* Behavioral Ecology and Sociobiology, 57, 438-444.
- Valerie, T. 2023. Do roosters have penises? How did they lose Them?
- Verrier, E., Danchin-Burge, C., Moureaux, S., Ollivier, L., Tixier-Boichard, M., Maignel, M.J., Bidanel, J.P., Clement, F., 2003. What should be preserved: genetic goals and collection protocols for the French National Cryobank. In: D. Planchenault (ed.), Workshop on Cryopreservation of

Animal Genetic Resources in Europe, Bureau des Resources Génétiques, Paris, France, pp. 79–89

- Villaverde-Morcillo, S., M. C. Esteso, C. Casta.o, and J. Santiago-Moreno. 2016. Influence of post-mortem sperm recovery method and extender on unstored and refrigerated rooster sperm variables. Reprod. Domest. Anim. 51:40–46.
- Vizcarra, J. A., N. Cerón-Romero, N. Taofeek, and J. Kirby. 2022. Reproduction in male birds (CG Scanes and S Dridi, Eds.). Academic Press.
- Wakayama, S., D. Ito, E. Hayashi, T. Ishiuchi, and T. Wakayama. 2022. Healthy cloned offspring derived from freeze-dried somatic cells. Nat. Commun. 13:3666.
- Wallace R.A., Selman K., 1981. Cellular and dynamic aspects of oocyte growth in teleosts. American Zoologist, 21, 325-343.
- Wildt, D. E., Comizzoli, P., Pukazhenthi, B., & Songsasen, N. (2010). Lessons from biodiversity—the value of nontraditional species to advance reproductive science, conservation, and human health. Molecular Reproduction and Development: Incorporating Gamete Research, 77(5), 397-409.
- Woelders, H., Zuidberg, C. A., & Hiemstra, S. J. (2006). Animal genetic resources conservation in the Netherlands and Europe: poultry perspective. Poultry science, 85(2), 216-222.
- Wojtczak M., Dietrich G.J., Słowińska M., Dobosz S., Kuźmiński H., Ciereszko A., 2007. Ovarian fluid pH enhances motility parameters of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Aquaculture, 270, 259-264.
- Wood C.D., Nishigaki T., Tatsu Y., Yumoto N., Baba S.A., Whitaker M., et al., 2007. Altering the speract-induced ion permeability changes that generate flagellar Ca2þ spikes regulates their kinetics and sea urchin sperm motility. Developmental Biology, 306, 525-537.
- Yamamoto K., 1963. Cyclical changes in the wall of the ovarian lumen in the medaka. Annotationes Zoologicae Japonenses, 36, 179-86.
- Yeates, S. E., Diamond, S. E., Einum, S., Emerson, B. C., Holt, W. V., & Gage, M. J. (2013). Cryptic choice of conspecific sperm controlled by the impact of ovarian fluid on sperm swimming behavior. Evolution, 67(12), 3523-3536.
- Yu, S., Kojima, N., Hakomori, S. I., Kudo, S., Inoue, S., & Inoue, Y. (2002). Binding of rainbow trout sperm to egg is mediated by strong carbohydrate-to-carbohydrate interaction between (KDN) GM3 (deaminated neuraminyl ganglioside) and Gg3-like epitope. Proceedings of the National Academy of Sciences, 99(5), 2854-2859.
- Zadmajid V., Myers J.N., Sørensen S.R., Butts I.A.E., 2019. Ovarian fluid and its impacts on spermatozoa performance in fish: a review. Theriogenology. 132, 144–152.

- Zakariah, M., R. A. Molele, M. A. A. Mahdy, M. I. A. Ibrahim, and L. J. McGaw. 2022. Regulation of spermatogenic cell apoptosis by the pro-apoptotic proteins in the testicular tissues of mammalian and avian species. Anim. Reprod. Sci. 247:107158.
- Zee, Y. P., W. V. Holt, J. Gosalvez, C. D. Allen, V. Nicolson, M. Pyne, M. Burridge, F. N. Carrick, and S. D. Johnston. 2008. Dimethylacetamide can be used as an alternative to glycerol for the successful cryopreservation of koala (Phascolarctos cinereus) spermatozoa. Reprod. Fertil. Dev. 20:724–733.
- Zhang, Y., Feng, B., Xie, Z., Deng, X., Zhang, M., Xie, Z., & Wang, S. (2020). Epidemiological surveillance of parvoviruses in commercial chicken and turkey farms in Guangxi, Southern China, during 2014–2019. Frontiers in Veterinary Science, 7, 561371.

Session 1

Improving fertilisation success: insights from ovarian fluid studies in Mediterranean trout (*S. Cetti*)

PREFACE

In Italy, although taxonomy remains controversial, currently the Mediterranean trout is designated as *Salmo cettii* according to the Habitat Directive Article 17. This is a trout species primarily found in Corsica, Sardinia, Sicily, and along the Adriatic and Tyrrhenian coasts of the Italian peninsula, including the Molise rivers. The Mediterranean trout, distributed in various areas of the national territory, exhibits significant biological diversity, mainly highlighted by genetic and phenotypic variation among populations. This diversity complicates the definition of morphology and specific color patterns for *Salmo cettii*. Traditionally, typical characteristics of *S. cettii (= S. macrostigma)* included small size, stout body, parr marks even in adults, a large preopercular spot and the absence of red spotting (Gandolfi et al., 1991). In the 1990s, common features were identified in native Apennine populations, including the presence of preopercular and parr marks even in adults, along with conspicuous red spotting. In recent years, the typical morphologies and color patterns of the Mediterranean trout have been updated. In its wide distribution range, Mediterranean trout shows extreme phenotypic diversity and considerable variation in biological characteristics (Esposito et al., 2022; D'Agaro et al., 2022). Indigenous trout from the Molise rivers (Biferno and Volturno) display four distinct tiger bands, black and/or red spotting (related to environmental features), more than nine parr marks in juveniles, and a consistently visible preopercular spot with a blue hue. The spawning period varies among the populations inhabiting the different Italian regions. Over recent centuries, the distribution range of native Mediterranean trout has undergone gradual shrinkage. Indeed, according to the Italian report, the conservation status of *S. cettii* in the Mediterranean biogeographic area is considered "unfavorable/poor" (U2) and "declining", and even "critically endangered" in the Italian IUCN Red List of Vertebrates (Bianco et al., 2013). This can largely be attributed to harmful anthropogenic interventions such as dam construction, river channelization, poaching, overfishing, and local pollution (Kottelat and Freyhof, 2007; Duchi, 2016). Additionally, in recent decades, activities related to recreational fishing have led to the introduction of domestic Atlantic strains capable of hybridizing with native populations (Penserini et al., 2005) in Italian and European watercourses, including those in the river basins of the Volturno and Biferno. Introgression from farmed strains has caused genetic impoverishment, removing original genetic variability and ecological specializations that are distinctive features of salmonid species. Therefore, it is considered one of the most serious threats to the long-term conservation of diversity within this species. Consequently, over the past two decades, native salmonid species, including *S. cettii*, have been the subject of significant conservation projects due to population decline and extinction in many countries. (Crivelli et al., 2000; Araguas et al., 2008, 2009; Carmona-Catot et al., 2010; Caudron et al., 2011; Caputo Barucchi et al., 2015; Sabatini et al., 2018; Lorenzoni et al., 2019). However, none

of these projects have so far involved the establishment of a semen bank for preserving the biodiversity of these important genetic resources.

In conservation projects, cryobanks represent indispensable tools for preserving the genetic diversity of various species, offering a crucial opportunity to support artificial reproduction practices and improve the genetic variability of offspring. This is particularly significant in the context of aquatic species, where sperm cells play a central role in cryopreservation efforts due to their compact size and greater resistance to cooling, making sperm cryopreservation more practical and effective than methods applied to other cell types.

In the situation, the European project "LIFE Nat.Sal.Mo" (https://greenproject.info/wpg/natsalmo/) was launched to ensure the recovery and conservation of native Mediterranean trout (*Salmo macrostigma* = *Salmo cettii*) in the river basins of Molise (Southern Italy) through the application of innovative techniques and participatory governance tools.

In this project, alongside habitat protection and restoration efforts, a pivotal objective is the restoration of the native trout's genetic integrity. This was made achievable through the establishment of a semen cryobank for this native trout species. A fundamental prerequisite for creating the first European semen cryobank dedicated to preserving native Mediterranean trout (*S. cettii*) from the rivers of Molise was the development of a sperm cryopreservation protocol. This milestone achievement was a crucial aspect of the LIFE Nat.Sal.Mo. project. The primary application of this cryobank lies in its integration into artificial reproduction practices, where doses of frozen semen from native males are used in combination with appropriate cross-fertilization schemes. The goal is to maximize offspring genetic diversity and mitigate genetic introgression within native trout populations in the project area. However, the success of fertilization depends on the swimming performance of sperm, highlighting the critical role of a suitable reproductive microenvironment.

In this context, the choice of an effective activation medium is crucial in artificial reproduction protocols, especially when using cryopreserved sperm.

Taking the aforementioned factors into account, the research activities outlined in this section encompass two studies, which are integral components of a broader project (LIFE Nat.Sal.Mo) coordinated by the University of Molise.

These efforts concentrate on enhancing fertilization success through artificial reproduction methods and advancing mechanisms involved in natural reproduction. To this end, the influence of ovarian fluid (OF) on both cryopreserved and fresh semen was investigated through *in vitro* experimentation.

In particular, the first study aimed at investigate the effect of ovarian fluid as a natural sperm activation medium on post-thaw swimming performance of Mediterranean trout sperm, comparing it with activating solution (D-532) and a solution composed of 50% D-532 and 50% ovarian fluid.

This study proposes that the presence of ovarian fluid, either alone or combined with D-532 in the artificial reproduction microenvironment, is a crucial factor in enhancing fertilization success when utilizing frozen semen of Mediterranean brown trout. Remarkably, OF 100% serves as a natural and effective medium, simplifying and expediting field operations conducted along riverbanks.

In the second study, the objective was to evaluate the potential capacity of ovarian fluid from native trout compared to ovarian fluid from non-native trout (*S. trutta)* on the swimming performance of sperm from native males inhabiting the Molise rivers.

In externally fertilizing fish, ovarian fluid (OF) appears to be crucial for fertilization success, enhancing the swimming performance of spermatozoa. These OF/sperm interaction mechanisms are frequently species-specific and/or population-specific and may reduce the likelihood of genetic introgression into wild populations from introduced or escaped zootechnical individuals.

The objective of our study was to investigate whether native *S. cettii* females produce ovarian fluid (OF) with a greater capacity to enhance sperm motility in conspecific males compared to zootechnical S. trutta females. To achieve this, we conducted a comparison of sperm swimming performance in males from the Biferno river (Molise region—Southern Italy) when activated by OFs from native *S. cettii* versus zootechnical *S. trutta* females. Our findings reveal that the OFs of native females (diluted by 20%) significantly enhance the sperm performance of autochthonous males, unlike those of zootechnical S. trutta. These initial findings indicate that interactions between OF and sperm could potentially influence or regulate hybridization mechanisms involving the native Mediterranean trout population in the Biferno river and the introduced domestic lineage of brown trout.

In conclusion, the two studies conducted as part of the LIFE Nat.Sal.Mo project have significantly enhanced our understanding of fertilization practices, encompassing both artificial and natural methods, crucial in Mediterranean trout conservation efforts. These findings pave the way for the identification of more efficient conservation strategies aimed at preserving the biodiversity and stability of native trout populations in the rivers of Molise.

Figure 1- Trout from Molise's rivers (Biferno and Volturno)

References

- Araguas, R.M.; Sanz, N.; Fernàndez, R.; Utter, F.M.; Pla, C.; Garcìa-Marìn, J.L. Genetic refuges for a self-sustained fishery: Experience in wild brown trout populations in the eastern Pyrenees. Ecol. Freshw. Fish 2008, 17(Suppl. 4), 610–616.
- Bernatchez L., 2001. The evolutionary history of brown trout (*Salmo trutta L.*) Inferred from phylogeographic, nested clade, and mismatch analyses of mitochondrial dna variation. Evolution, 55, 351-379.
- Bernatchez L., Guyomard R., Bonhomme F., 1992. DNA sequence variation of the mitochondrial control region among geographically and morphologically remote European brown trout Saltno trutta populations. Molecular Ecology, 1, 161–173. 46
- Bianco, P.G.; Caputo, V.; Ferrito, V.; Lorenzoni, M.; Nonnis Marzano, F.; Stefani, F.; Sabatini, A.; Tancioni, L. Pesci d'acqua dolce, in: Rondinini, C., Battistoni, A., Peronace, V., Teofili, C (Eds), Lista Rossa IUCN dei Vertebrati Italiani. Comitato Italiano IUCN e Ministero dell'Ambiente e della Tutela del Territorio e del Mare, Roma, 2013, pp. 54. Brook trout remova as a conservation tool to restore eagle lake rainbow trout. N. Am. J. Fish. Manag. 2010, 30, 1315–1323.
- Caputo Barucchi, V.; Carosi, A.; Giovannotti, M.; La Porta, G.; Splendiani, A.; Lorenzoni, M. Life+ Trout Project (LIFE12 NAT/IT/0000940) for the recovery and conservation of Mediterranean trout (*Salmo trutta complex*) in the central Apennines (Italy). XV European Congress of Ichthyology 7-11 September 2015. Porto (Portugal).
- Carmona-Catot, G.P.; Moyle, B.; Aparicio, E.; Crain, P.K.; Thompson, L.C.: García-Berthou, E. Brook trout removal as a conservation tool to restore eagle lake rainbow trout. N. Am. J. Fish. Manag. 2010, 30, 1315–1323
- Caudron, A.; Champigneulle, A.; Guyomard, R.; Largiadèr, C.R. Assessment of three strategies practiced by fishery managers for restoring native brown trout *(Salmo trutta)* populations in Northern French Alpine Streams. Ecol. Freshw. Fish 2011, 20, 478–491.
- Conservation status of habitat types and species Conservation. Article 17. Habitat Directive 92/43/CEE. Available: https://data.europa.eu/euodp/it/data/dataset/data_article-17-databasehabitats- directive-92-43-eec-1.
- Crivelli, A.J.; Poizat, G.; Berrebi, P.; Jesensek, D.; Rubin, J.F. Conservation biology applied to fish: The example of a project for rehabilitating the Marble trout *(Salmo marmoratus)* in Slovenia. Cybium 2000, 24, 211–230.

- Duchi, A. The combination of a biotic and a chemical-microbiological index shows the environmental risks of a high quality Mediterranean stream (Tellesimo; S.C.I. ITA090018; Sicily, Italy). Int. J. Innov. Res. Sci. Eng. Technol. 2016, 5, 6630–6637.
- Gandolfi G., Torricelli P., Marconato A., Zerunian S., 1991. I pesci delle acque interne Italiane. Istituto Poligrafico e Zecca dello Stato, Roma, XVI, 617.
- Gibertoni P.P., 2005. "Valorizzazione della fauna ittica autoctona nelle acque del Parco Regionale di Crinale dell'Appennino Reggiano". Parco del Gigante (RE).
- Iaffaldano N., Di Iorio M., Manchisi A., Esposito S., Gibertoni P.P., 2016a. Effective freezing rate for semen cryopreservation in endangered Mediterranean brown trout (*Salmo trutta macrostigma*) inhabiting the Biferno river (South Italy). Zygote, 24 (5), 668-675.
- Kottelat, M.; Freyhof, J. Handbook of European freshwater fishes. Kottelat, Cornol, Switzerland and Freyof, Berlin, Germany 2007.
- Lorenzoni, M.; Carosi, A.; Giovannotti, M.; La Porta, G.; Splendiani, A.; Caputo-Barucchi, V. Ecology and conservation of the Mediterranean trout in the central Apennines (Italy). J. Limnol. 2018, 78(1), 1-13.
- Penserini, M.; Nonnis, M.F.; Gibertoni, P.P.; Maldini, M. Introgressione in popolazioni autoctone di salmo trutta indetificata mediante marcatori nucleari e mitocondriali. Tesi di Laurea in Scienze Naturali – Dipartimento di Biologia Evolutiva e Funzionale – Università di Parma. 2005.
- Sabatini, A.; Podda, C.; Frau, G.; Cani, M.V.; Musu, A.; Serra M.; Palmas F. Restoration of native Mediterranean brown trout *Salmo cettii* Rafinesque, 1810 (Actinopterygii: Salmonidae) populations using an electric barrier as a mitigation tool. Eur. Zool. J. 2018, 85(1), 138–150.

STUDY 1

Veterinary Science 2023, 10(3), 219.
(https://doi.org/10.3390/vetsci10030219)

veterinary sciences

MDPI

Article

The Use of Ovarian Fluid as Natural Fertilization Medium for Cryopreserved Semen in Mediterranean Brown Trout: The Effects on Sperm Swimming Performance

Giusy Rusco [1], Michele Di Iorio [1], Stefano Esposito [2], Emanuele Antenucci [1], Alessandra Roncarati [3] and Nicolaia Iaffaldano [1,*]

[1] Department of Agricultural, Environmental and Food Sciences, University of Molise, 86100 Campobasso, Italy
[2] Mediterranean Trout Research Group "MTRG", 42032 Ventasso (RE), Italy
[3] School of Biosciences and Veterinary Medicine, University of Camerino, 62024 Matelica, Italy
* Correspondence: nicolaia@unimol.it; Tel.: +39-0874-404697

Simple Summary: In the context of the "Life—Nat.Sal.Mo" project, obtaining an effective semen cryopreservation protocol was an important milestone that allowed the implementation of the first European cryobank of native Mediterranean brown trout (*S. cettii*) inhabiting Molise rivers (Italy). The main use of our semen cryobank is represented by its practical application in artificial reproduction practices aimed at maximizing the genetic variability of the offspring and reducing the genetic introgression in the native trout populations in the project area. Thus, the choice of the most suitable activation/fertilization medium represents the last key step in the development of artificial reproduction protocols using cryopreserved semen. Therefore, the aim of the present study was to investigate the effect of ovarian fluid as a natural activation media of spermatozoa on the post-thaw sperm swimming performance of Mediterranean trout, comparing it with D-532 and a mixed solution of 50% D-532 and 50% ovarian fluid. Our results suggest that the presence of ovarian fluid alone or in combination with D-532 in the artificial microenvironment of reproduction represents a key factor to increase the success of fertilization when the frozen semen of Mediterranean brown trout is used.

Abstract: D-532 fertilization solution is generally used to replace the water or ovarian fluid during artificial reproductive practices in salmonids due to its ability to boost sperm motility and increase fertilization rates compared with natural activation media. However, the maintenance of ovarian fluid in a reproductive microenvironment gives it the advantage of protecting the eggs from potential harmful factors from the external environment and simplifying the field operations related to its removal when D-532 is used alone. In light of this, the aim of the present study was to investigate in vitro, for the first time, the effect of ovarian fluid (OF 100%) on post-thaw sperm swimming performance of Mediterranean trout, comparing it with D-532 and a mixed solution of 50% D-532 and 50% ovarian fluid (OF 50%). The percentage of motile spermatozoa and movement duration was significantly increased in OF 100% and OF 50% compared with D-532. Sperm velocity was higher in D-532, but significant differences were recorded only with OF 100%. In conclusion, these results suggest that the presence of ovarian fluid alone or in combination with D-532 in an artificial microenvironment of reproduction represents a key factor in potentially increasing fertilization success when the frozen semen of Mediterranean brown trout is used.

Keywords: fertilization medium; frozen semen; artificial reproductive microenvironment; conservation project

check for updates

Citation: Rusco, G.; Di Iorio, M.; Esposito, S.; Antenucci, E.; Roncarati, A.; Iaffaldano, N. The Use of Ovarian Fluid as Natural Fertilization Medium for Cryopreserved Semen in Mediterranean Brown Trout: The Effects on Sperm Swimming Performance. *Vet. Sci.* **2023**, *10*, 219. https://doi.org/10.3390/vetsci10030219

Academic Editor: Fred Sinowatz

Received: 27 January 2023
Revised: 1 March 2023
Accepted: 9 March 2023
Published: 13 March 2023

Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

In the context of conservation projects, cryobanks are a valuable tool to preserve the genetic resources of a wide range of species, providing a crucial opportunity to support the artificial reproduction practices aimed to increase the genetic variability of offspring (Roldán and Garde, 2004; Martínez-Páramo, et al. 2009, 2017). Sperm cells are the main type of cells used for cryobanking purposes in aquatic species. In fact, thanks to their small size and relatively high resistance to chilling, sperm cryopreservation is a more feasible method compared with the ones performed on other cell types (Martínez Páramo et al., 2017). The obtainment of an effective semen cryopreservation protocol was an important milestone in our project (LIFE Nat.Sal.Mo.), that allowed us to implement the first European cryobank of the native Mediterranean brown trout (*S. cettii*) inhabiting Molise rivers. (Rusco et al., 2020; Di Iorio et al., 2023). The main use of our semen cryobank is represented by its practical application in artificial reproduction practices. Indeed, frozen semen doses of native males are used in cross-fertilization schemes within artificial reproduction to maximize the genetic variability of offspring and reduce the genetic introgression in the native trout populations within the project area. An important prerequisite for successfully fertilizing eggs is the sperm swimming performance. In this regard, it is known that an appropriate reproductive microenvironment is crucial to promote a good activation of sperm motility and their simultaneous encounter with eggs; this result is particularly important in external fertilizer fish (Alavi et al., 2005, 2006;). For this reason, the selection of the most suitable activation/fertilization medium represents a crucial step in the artificial reproduction protocols, especially when cryopreserved semen is adopted. Generally, artificial fertilization media are developed in order to boost sperm motility and increase fertilization rates compared with natural media, such as water or ovarian fluid (Wilcox et al., 1984, Billard, 1988,1992, Goetz and Coffman, 2000; Dietrich et al., 2007; Hugunin et al.,2008; Hatef el al., 2009; Łuczynski el al., 2022). In particular, among more effective artificial fertilization media, D-532 saline solution was used to replace ovarian fluid during the artificial fertilization practices in salmonids in order to avoid the variability problems related to the composition of ovarian fluids and their possible contamination with yolk from broken eggs (Billard et al., 1992). In this regard, in our previous studies aimed at fine-tuning the semen cryopreservation protocols for native Mediterranean brown trout (Di Iorio et al., 2023; Rusco et al., 2021), we used D-532 solution for the *in vivo* fertilization trials in order to reduce the variability of the results due to the differences in the quality of the ovarian fluid from individual females as much as possible, making the microenvironment of fertilization more homogeneous. Nevertheless, in subsequent fertilization practices with frozen sperm performed within the project Nat.Sal.Mo. on the river bank, ovarian fluid alone as a natural activation medium of sperm motility was used

for two main reasons: (1) to maintain the artificial microenvironment of reproduction as similar as possible to that of natural spawning, protecting the eggs from potential harmful factors from the external environment; (2) to reduce the time required for the complete removal of ovarian fluid when D-532 is used alone. Surprisingly, we noted that the use of ovarian fluid alone registered generally higher fertilization rates (Di Iorio et al., 2023) than those we found during the in vivo trials conducted in the presence of D-532 (Rusco et al., 2020, 2021).

The ability of ovarian fluid to significantly affect the swimming performance of fresh sperm and consequently influence the outcome of fertilization in terms of fertilized oocyte rate has been shown in several fish species (Hatef et al., 2009; Turner et al., 2002; Urbach et al., 2005; Rosengrave et al., 2008,2009,2016; Dietrich et al., 2008; Diogo et al., 2010; Butts et al., 2012, 2017; Galvano et al., 2013; Alonzo et al., 2016; Zadmajid et al., 2019; Poli et al., 2019). However, to the best of our knowledge, there are no studies that have tested the effect of ovarian fluid on the motility parameters of frozen semen.

In light of these considerations and given the optimal fertilization rates obtained within our project using ovarian fluid alone, we investigated, for the first time, the in vitro effect of ovarian fluid on the post-thaw sperm of Mediterranean trout's swimming performance and compared it with D-532 fertilization solution. Moreover, a mixed solution that included 50% D-532 and 50% ovarian fluid was also tested to evaluate the potential use of this combined medium in order to avoid the elimination of ovarian fluid, simplifying the field operations.

2. Materials and Methods

2.1. Animals and Collection of Samples

During the breeding season (January–February 2021), 17 breeders (7 males and 10 females) of native Mediterranean brown trout were captured in the Biferno river by electrofishing. The males belonged to the 2+ and 3+ classes, with an average total length of 30.2 ± 3.9 cm; the females were 3+ and 4+ years old and characterized by an average total length of 44.1 ± 5.2 cm. Semen samples (N = 7) were collected by gentle abdominal massaging, drying the abdomen and urogenital papilla with special care before stripping in order to avoid contamination of semen with urine, mucus and blood cells.

Eggs with their own ovarian fluids (OFs) were collected by the same method described above for males, and ovarian fluids were separated from egg batches of each female (N = 10) directly with a syringe after egg decantation.

After sample collection, both males and females were immediately released into the water course. The sperm and OF samples were transferred from the river to the laboratory in a cooler that

contained ice and processed within 4 h of the collection. Semen samples were subjected to the cryopreservation procedure optimized in our previous work (Rusco et al., 2020).

Briefly, each semen aliquot was diluted with a freezing extender to reach 0.15M of glucose, 7.5% of methanol and a sperm concentration of 3.0×10^9 sperm/mL. The diluted semen was charged into 0.25 mL plastic straws and equilibrated for 15 min on ice (at the height of 3 cm). Lastly, the straws were cryopreserved by exposure to liquid nitrogen (LN_2) vapor at 3 cm above the LN_2 level for 5 min and plunged into LN_2.

The OFs were subjected to pH measurement using a BasiC 20 pH meter (CRISON instruments, Barcelona, Spain) and subsequently stored at −20 C until osmolality measurement by an OSMOMAT 3000 basic (GONOTEC, Reuchlinstr, Berlin, Germany), according to the manufacturer's protocol. OF samples were frozen because of the impossibility to collect all of the sperm and ovarian fluid samples on the same day (Purchase et al., 2020;Cosson et al., 2010).

2.2. Experimental Design

Sperm motility analyses were performed using a Computer-Assisted Sperm Analysis (CASA) system coupled to a phase contrast microscope (Nikon model Ci-L) employing the Sperm Class Analyzer (SCA) software (VET Edition, Barcelona, Spain), that carried out two replicate sperm activations for each treatment combination.

The experiment was conducted over 11 days. On the 1st day, the semen samples (N=7) were analyzed after thawing at 40 °C for 5 s using two different activation media: 1% $NaHCO_3$ as a control solution (C) to test cryopreserved semen quality and D-532 as a benchmark artificial fertilization solution (20 mM Tris, 30 mM glycine, 125 mM NaCl, pH 9.0), at a dilution ratio of 1:30 (*v:v*). On each of the remaining 10 days, one microtube of OF from a single female (N=10) was thawed at 4°C for 2 h and split into 2 aliquots: one was used as OF 100%, and the other diluted at 50% with the D-532 fertilization solution (OF 50%). Subsequently, the thawed semen from each male was activated in OF 100% and OF 50%, using the same dilution ratio reported above. After rapid mixing, 3 μL of diluted semen was immediately loaded onto a 20-micron Leja slide (Leja Standard Count, Nieuw Vennep, The Netherlands) and evaluated by the computer-assisted sperm analysis system (CASA). Sperm motility was analyzed 10 s post-activation, acquiring videos at 25 fps rate. The following sperm traits were evaluated: motile spermatozoa (MOT, %), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, %), straightness (STR, %), beat cross frequency (BCF, Hz) and amplitude of lateral displacement of the spermatozoon head (ALH, μm). The duration of sperm movement (DSM) was measured using a chronometer from sperm activation to movement cessation of spermatozoa in

the field of view. The loading of chambers and the recording of motility parameters were both carried out by the same operator, taking care to standardize the operation timing from ice to observation.

Sperm motility is nowadays considered the best sperm quality biomarker in fish as they are strongly correlated with fertilization ability. In this regard, the CASA system is the most objective and comprehensive quantification method currently available to assess several sperm motility parameters in various species, including fish (Wilson-Leedy et al., 2007).

2.3. Statistical Analysis

All statistical analyses were performed using the statistical software R (Version 4.2.0), at significance levels of $p \leq 0.05$. The replicates were treated as repeated measures, using its mean for statistical analysis. MOT (%) measurements were subjected to prior *arcsine* transformation. The correlations among sperm motility parameters were analyzed in order to identify closely related parameters and reduce the number of parameters to be addressed and discussed.

Normality and homoscedasticitywere tested by visual inspection of the residuals' graphs, Shapiro–Wilk test and Levene's test (*rstatix* and *car* R packages). The differences among treatments for all tested sperm motility parameters were analyzed usingMixedModel ANOVAs with the fertilization medium as a fixed factor and the ID male as a random factor, followed by Tukey's post-hoc test for multiple comparisons between treatments (*lme4*, *lmerTest* and *multcomp* R packages). Furthermore,MixedModel ANOVAs were conducted, isolating the OF groups to test the differences between OF 100% and OF 50%, adding the random effect of females, males and their interaction. The AIC test was used to compare different models and determine which one was the best fit for the data (*lmerTest* R package).

To evaluate the association between the quality of cryopreserved semen assessed through the activation in C and its performance in the fertilization media, the correlations between sperm motility parameters registered in OFs (100% and 50%) and in the control activation solution (C) were tested using the Pearson parametric test (*Hmisc* and P*erformanceAnalytics* R packages).

All graphics were generated using the *ggplot2* package.

3. Results

The average (±SD) osmolality and pH of the ovarian fluids (N=10) were 234.7 ± 74.77 mOsm/kg and 8.63 ± 0.17, respectively. The three velocities (VCL, VAP and VSL) and two linearity parameters (STR and LIN) resulted as strictly associated. Therefore, we decided to reduce the set of showed and discussed parameters to six (MOT, VCL, LIN, ALH, BCF, DSM). All sperm

motility parameters and the correlation matrix are reported in Supplementary Material (Table S1 and Figure S1).

3.1. Comparison of the Post-Thaw Sperm Motility Parameters among the Different Activation Media

The significant differences among the post-thaw sperm motility parameters activated in OF 100%, OF 50% or D-532 are shown in Table 1. The activation solutions that included the ovarian fluids (OF 100% and OF 50%) induced a significant increase in total sperm motility (MOT) and its duration (DSM) compared with D-532. However, a significant increase in VCL was observed using D-532 and OF 50% with respect to OF 100%. Linearity (LIN) was higher when spermatozoa were activated in D-532. Furthermore, significantly higher values of lateral displacement amplitude of the spermatozoon head (ALH) were found for cryopreserved semen activated in OF 50%. No differences among different treatments for BCF were found. In Figure 1, the variability range of motility parameters is shown. A wider variability is observed when ovarian fluid is used.

3.2. Differences between OF 100% and OF 50%: The Effect of Males, Females and Male–Female Interaction

The results for the best-fitting mixed models are reported in Table 2. For MOT, VCL and ALH, a significant difference was found between OF 100% and OF 50%. In particular, MOT and ALH were significantly higher in OF 50%, with an interaction effect between males and females. VCL was also higher in OF 50%, with the best model represented by the only random effect of inter-male variability, according to pairwise comparison in Section 3.1 (Table 2). No differences were observed for LIN, BCF and DSM between OF 100% and OF 50%.

Table 1. Main sperm motility parameters (means ± SD) in D-532, OF 50% and OF 100% and pairwise comparison among treatments

Sperm Traits	Treatments		
	D-532	**OF 50%**	**OF 100%**
MOT (%)	43.04 ± 13.59 ^{b}	57.49 ± 14.00 ^{a}	55.56 ± 13.83 ^{ab}
VCL (μm/s)	138.01 ± 18.74^{a}	122.29 ± 32.01^{a}	107.72± 27.69^{b}
LIN (%)	64.23 ± 7.79^{a}	50.00 ± 8.55^{b}	49.98 ± 9.90^{b}
ALH (μm)	2.49 ± 0.57^{b}	3.21 ± 0.80^{a}	2.84 ± 0.70^{b}
BCF (Hz)	4.28 ± 0.72	3.71 ± 0.84	3.72 ± 0.94
DSM (s)	32.86 ± 6.33^{b}	46.39 ± 9.60^{a}	45.98 ± 10.84^{a}

^{ab}Different superscript letters within the same row indicate a significant difference ($p < 0.05$).

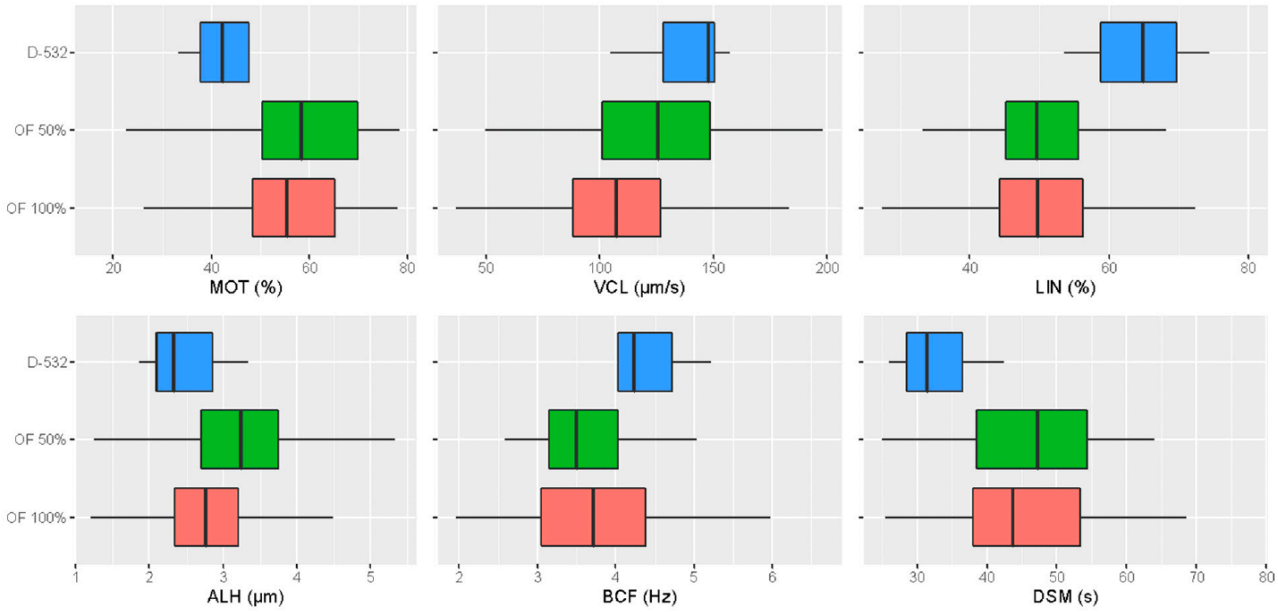


Figure 1. Boxplots displaying the "within" and "between" treatments variability concerning the main sperm motility parameters (outliers are not shown)

Table 2- Selection of the best-fitting model for mixed model ANOVAs isolating the of groups, with the treatment (OF 100% *vs.* OF 50%) as a fixed factor and male, female or their interaction as random factors. only the parameters with significative differences among treatments are shown.

Random Effects	**MOT**		**VCL**		**ALH**	
	p	AIC	*p*	AIC	*p*	AIC
Male	***	–154.51	***	1308.1	**	320.14
Female	*	–129.15		1346.2	**	320.5
Male-Female	***	–216.94	***	1329.2	***	309.83
Fixed effect						
Treatment (OF 100% vs. OF 50%)	*		***		***	

$p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***. The best-fitting models (lowest AIC values) are marked in bold

3.3. Correlation among the Post-Thaw Sperm Motility Parameters Activated in Ovarian Fluid and Control Activation Solution

The MOT, VCL, BCF and DSM values recorded using OF (OF 50% and OF 100%) were positively correlated with those obtained in the control activation solution (C). No relationship was identified for LIN and ALH (Figure 2).

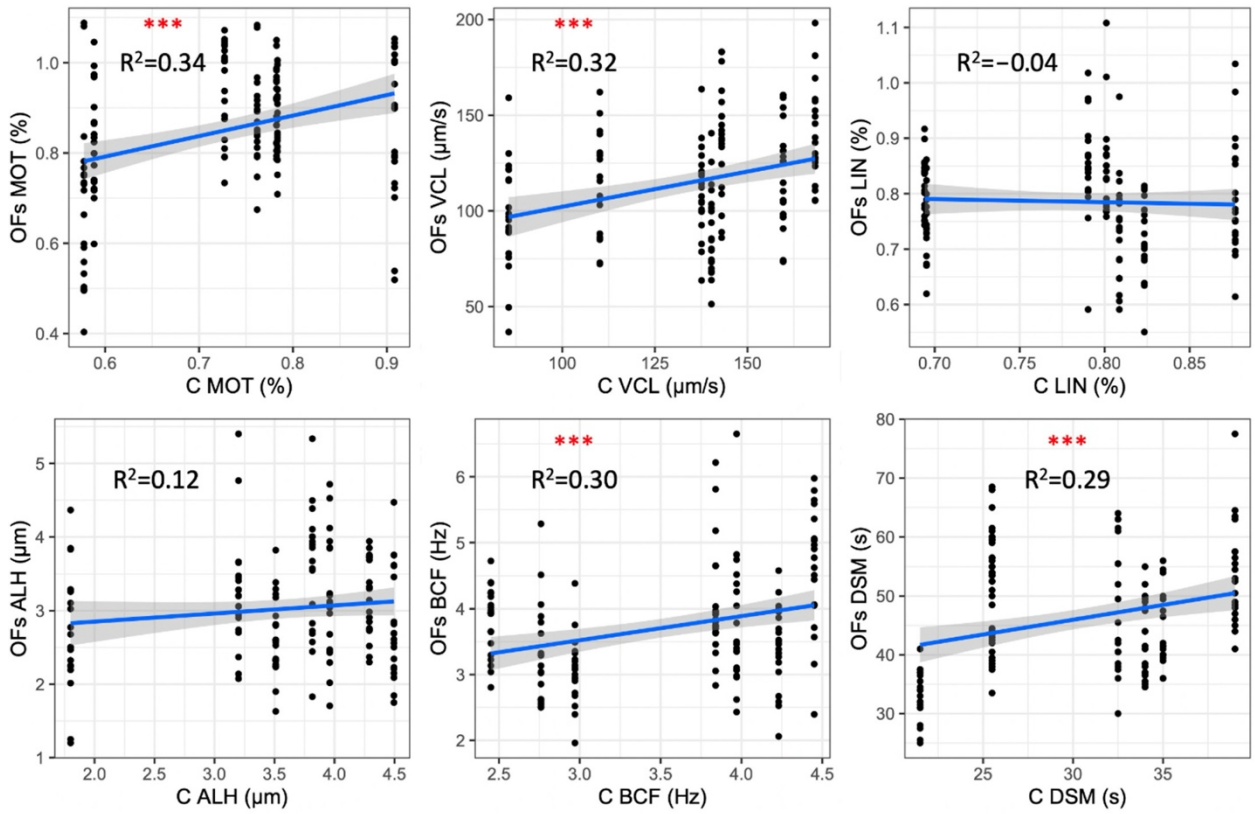


Figure 2- Correlation among the post-thaw sperm motility parameters using Ovarian Fluid and control activation solution. Significance levels (in red) and coefficients of determination (R^2) are reported. ***: $p < 0.001$.

4. Discussion

For the first time, the effect of ovarian fluid on the post-thaw sperm motility parameters of Mediterranean brown trout *S. cettii*[1] was tested, and it was compared both with an artificial fertilization medium (D-532) and ovarian fluid supplemented with 50% D-532.

Our results showed that the activation solutions with ovarian fluid (OF 100% and OF 50%) significantly prolonged the duration of movement (longevity) compared with D-532 alone, confirming the on-field observation of the fertilization success obtained during the LIFE Nat.Sal.Mo. project (Di Iorio et al.,2023).

It is possible that some chemical constituents of ovarian fluid influence ATP metabolism, such that the duration of energy production is increased (Turner et al.,2002). Total motility was also higher in OF 100% and OF 50%; however, significant differences were found only between OF 50% and D-532. On the contrary, when D-532 was used, the sperm velocity parameters underwent an overall

[1] Mediterranean brown trout is listed in Annex II of the Habitat Directive as *Salmo macrostigma* and currently reported as *Salmo cettii* in the Italian and European Red Lists and in the Member States' conservation status assessments of the "habitats types and species of Community interest" (Art. 17). Recent studies suggest that Italian peninsular Mediterranean brown trout belong to a separate taxon named *S. ghigii*, limiting the name *S. cettii* to Sicilian trout (Lorenzoni et al., 2019; D'Agaro et al., 2022; Polgar et al., 2022). Although we agree with the recent observations, we still use the name *S. cettii* in the current paper, because Mediterranean brown trout populations are still protected by the Habitat Directive and subsequent updates on its conservation status.

increase at the potential expense of longevity. It is, in fact, possible that a higher swimming velocity required higher energy consumption by spermatozoa, thus leading to a lower movement duration, as suggested by Cosson et al., 2010. In addition, from these results, it is interesting to note the potential positive effect exercised by the combination of ovarian fluid and D-532 (OF 50%) on the post-thaw sperm motility parameters. The presence of D-532 solution in the OF, in fact, significantly increased the velocity parameters compared with OF alone; at the same time, the presence of OF in D-532 solution significantly improved both the total motility and the duration of movement in comparison to the D-532 buffer alone. The prolongation of the duration of sperm movement obtained by adding 50% of ovarian fluid to the D-532 buffer is consistent with findings found by Dietrich et al. 2005 using fresh semen from rainbow trout (*Oncorhynchus mykiss*). The high standard deviation registered for some parameters is explained by differences between males (the random factor in our mixed model). Furthermore, we observed that the presence of ovarian fluid in the activation media increased the variability of results (Figure 1), which could be mainly explained by the inter-female variability and/or the male x female interaction effect (Supplementary Material: Figures S2–S4). The female effect is generally attributed to the intrinsic differences in the composition of ovarian fluid from individual females, such as affecting sperm motility characteristics differently (Wojtczak et al., 2007; Beirão et al., 2014; İnanan et al.,2015). At the same time, the male effect is due to the different intrinsic sperm qualities existing among individual males. In this regard, some authors (Dietrich et al., 2008; Galvano et al., 2013;) suggested that some males produce sperm with superior motility characteristics despite the variability in the motility-modulating activity of particular ovarian fluid.

We found positive correlations for many of the sperm motility parameters (MOT, VCL, BCF, and DSM; Figure 2) among the solutions containing ovarian fluid (OF 100% and OF 50%) and the control solution, suggesting that sperm of good quality, once activated, moves with similar parameters regardless of the activation media. The correlations for MOT, VCL, BCF and DSM are all characterized by $p < 0.001$ and R^2 around 0.3. For such kinetic parameters, the combination of high significance levels and low R^2 suggests that the performances in OFs were significatively correlated with the control values (activation in C medium) registered for each male, but they do not explain a great part of the variability that most likely arises from females and/or male–female effects. Thus, these results show that the activation in the control solution is a good proxy for the swimming performance of post-thawed cryopreserved sperm in the fertilization environment.

However, the limited or negative effect of ovarian fluid on spermatozoa performance is often associated with the presence of contaminants, such as vitellus from broken eggs, blood, water, feces, etc. (Dietrich et al., 2017; Beirão et al., 2014; Hamano, S. et al., 1961; Nomura et al.,1964;

Billard et al., 1977; Aegerter et al., 2004). In this regard, in order to counteract the negative effects of contamination, some authors recommend to discard ovarian fluid from salmonid eggs before fertilization and to use only isotonic diluents of pH 8.4–9.0 to enhance sperm motility (Wilcox et al., 1984; Billard et al., 1992; Goetz and Coffman, 2000; Dietrich et al., 2007; Hugunin et al.,2008; Hatef el al., 2009). Nevertheless, the composition of ovarian fluid that includes ions, proteins, amino acids and sugar is ideal to support and protect the eggs and, at the same time, extend the fertilization period both in the natural and artificial fertilization environment (Billard et al., 1974,1983,1988; Lahnsteiner et al., 1995; Kholodnyy et al.,2020;). In light of this, we considered that leaving the ovarian fluid as natural sperm activation media during artificial reproduction practices carried out on the river- bank within our project was good practice. OF, as well as maintaining the advantage to maintain the artificial microenvironment of reproduction as similar as possible to that of natural spawning, allowed us to facilitate the management of field operations, avoiding the preparation, transport and the use of fertilization solutions. The use of D-532 alone would require the complete removal of ovarian fluid through a sieve; this time-consuming operation, in an external uncontrolled environment, exposes the eggs to possible freezing damage when temperatures are below zero; therefore, giving up the natural protection provided by the ovarian fluid.

Based on our results, the addition of a portion of D-532 to the eggs in the presence of their ovarian fluid could be another possible solution in order to facilitate field operations. However, we believe that this step is not indispensable because, although it is known that velocity is positively correlated with fertilization rates (Gage et al., 2004), it may not be a key parameter for successful fertilization when cross-fertilization schemes are adopted within artificial reproduction practices. From an application point of view, these fertilization schemes consist of splitting the eggs from each native female into equal aliquots so each of them can be fertilized with a frozen semen dose from different males. Therefore, a controlled reproductive microenvironment is created (in the absence of male competition) that already favors the gametes encounter. Moreover, since it is known that the cryopreservation process causes cell damage and compromises sperm motility and longevity (Figueroa et al., 2019), obtaining a greater number of post-thaw motile sperm/eggs that move for a longer time could potentially enhance fertilization success. To corroborate the above, higher fertilization rates, ranging from 64% to 81%, were achieved when only ovarian fluid was used (Di Iorio et al., 2023) compared with those obtained in our previous papers (ranging from 53% to 65%) by also adding to eggs, the D-532 buffer (Rusco et al., 2020,2021).

5. Conclusions

The use of cryobanks supporting the conservation of endangered native species could solve or mitigate the known problems affecting the supportive breeding and the management of broodstocks, contributing to maintain the wild genetic biodiversity and avoiding domestication. To extend the use of these practices, the definition of operational cryopreservation and fertilization protocols is crucial. In this regard, this study suggests that the presence of ovarian fluid alone or in combination with D-532 in the artificial microenvironment of reproduction represents a key factor to increase the success of fertilization when frozen semen of Mediterranean brown trout is used. Notably, OF 100% is a natural, viable media for simplifying and expediting the field operations that occur on riverbank.

In the future, it will be interesting to characterize the ovarian fluid biochemistry from native Mediterranean trout in order to investigate the components that can impact the in vitro and in vivo performance of fresh and frozen semen and to standardize fertilization protocols in controlled reproduction. Finally, the obtained results could be useful not only for the conservation of the native Mediterranean trout, but also to stimulate further research on the use of pure or mixed ovarian fluid as fertilization medium in other species of aquaculture interest.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vetsci10030219/s1, Table S1: All sperm motility parameters (means ± SD) in C, D-532, OF 50% and OF 100% and pairwise comparison among treatments; Figure S1: Correlation matrix among all the sperm motility traits; Figure S2: Barplots showing the additive male effect on total motility (MOT) and duration (DSM) grouping all treatments; Figure S3: The barplots show the additive female effect (mean of all male crosses) on total motility (MOT) and duration (DSM) grouping OF 50% and OF 100 %. Values registered in C and D.532 are shown; Figure S4: The barplot shows the interaction effect between male and female on total motility (MOT) grouped by male (each coloured bar) and female (each group of bars). Both females and male are sorted by their overall means.

Author Contributions: Conceptualization, N.I., G.R. and S.E.; Data curation, S.E., M.D.I. and G.R.; Formal analysis, G.R., M.D.I. and S.E.; Methodology, G.R., M.D.I. and E.A.; Writing—original draft, G.R. and S.E.; Writing—review and editing, N.I., A.R. and M.D.I.; Funding Acquisition, N.I. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the LIFE Nat.Sal.Mo. project (LIFE17 NAT/IT/000547).

Institutional Review Board Statement: The experiments were conducted in accordance with the Code of Ethics of the EU Directive 2010/63/EU for animal experiments. This study is part of a Nat.Sal.Mo LIFE project that received "a positive opinion" from the Ministry of the Environment and the Protection of the Territory and the Sea. The sampling and handling of fish followed animal welfare practices as reported in the Ministerial Protocol (ISPRA). All experiments were carried out with the appropriate authorizations from the Molise Region–Dipartimento Governo del Territorio, Mobilità e Risorse Naturali cod. DP.A4.02.4N.01 (protocol number 3969, 3 August 2018), according to the current regulations on the protection of the species, biosecurity, protocols of sampling of fresh water and animal welfare.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank Amber Burchell for the English language revision of the manuscript.

Conflicts of Interest: The authors declare that they have no competing interests.

References

1. Roldan, E.R.S.; Garde, J. Biotecnología de la reproducción y conservación de especies en peligro de extinción. In *Los Retos Medioambientales del Siglo XXI*; Gomendio, M., Ed.; La Conservación de la Biodiversidad en España, Fundación BBVA: Bilbao, Spain, 2004; pp. 283–307.
2. Martínez-Páramo, S.; Pérez-Cerezales, S.; Gómez-Romano, F.; Blanco, G.; Sánchez, J.A.; Herráez, M.P. Cryobanking as tool for conservation of biodiversity: Effect of brown trout sperm cryopreservation on the male genetic potential. *Theriogenology* **2009**, *71*, 594–604. [CrossRef] [PubMed]
3. Martínez-Páramo, S.; Horváth, A.; Labbé, C.; Zhang, T.; Robles, V.; Herráez, P.; Suquet, M.; Adams, S.; Viveiros, A.; Tiersch, T.R.; et al. Cryobanking of aquatic species. *Aquaculture* **2017**, *472*, 156–177. [CrossRef] [PubMed]
4. Rusco, G.; Di Iorio, M.; Iampietro, R.; Esposito, S.; Gibertoni, P.P.; Penserini, M.; Roncarati, A.; Iaffaldano, N. A Simple and Efficient Semen Cryopreservation Method to Increase the Genetic Variability of Endangered Mediterranean Brown Trout Inhabiting Molise Rivers. *Animals* **2020**, *10*, 403. [CrossRef] [PubMed]
5. Di Iorio, M.; Rusco, G.; Esposito, S.; D'Andrea, M.; Roncarati, A.; Iaffaldano, N. The role of semen cryobanks for protecting endangered native salmonids: Advantages and perspectives as outlined by the LIFE Nat.Sal.Mo. project on Mediterranean brown trout (Molise region—Italy). *Front. Mar. Sci.* **2023**, *9*, 1075498. [CrossRef]
6. Alavi, S.M.H.; Cosson, J. Sperm motility in fishes. I. Effects of temperature and pH: A review. *Cell Biol. Intern.* **2005**, *29*, 101–110. [CrossRef]
7. Alavi, S.M.H.; Cosson, J. Sperm motility in fishes. II. Effects of ions and osmolality: A review. *Cell Biol. Intern.* **2006**, *30*, 1–14.[CrossRef]
8. Wilcox, K.W.; Stoss, J.; Donaldson, E.M. Broken eggs as a cause of infertility of coho salmon gametes. *Aquaculture* **1984**, *40*, 77–87.[CrossRef]
9. Billard, R. Artificial insemination and gamete management in fish. Mar. *Freshw. Behav. Physiol.* **1988**, *14*, 3–21.

10. Billard, R. Reproduction in rainbow trout: Sex differentiation, dynamics of gametogenesis, biology and preservatic of gametes. *Aquaculture* **1992**, *100*, 263–298. [CrossRef]
11. Goetz, F.W.; Coffman, M.A. Storage of unfertilized eggs of rainbow trout (*Oncorhynchus mykiss*) in artificial media. *Aquaculture* **2000**, *184*, 267–276. [CrossRef]
12. Dietrich, G.J.; Wojtczak, M.; Słowińska, M.; Dobosz, S.; Kuźmiński, H.; Ciereszko, A. Broken eggs decrease pH of rainbow trout (*Oncorhynchus mykiss*) ovarian fluid. *Aquaculture* **2007**, *273*, 748–751. [CrossRef]
13. Hugunin, H.A.; Parsons, J.E.; Nagler, J.J. The influence of coelomic fluid on in vitro fertilization success in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **2008**, *281*, 155–157. [CrossRef]
14. Hatef, A.; Niksirat, H.; Alavi, S.M.H. Composition of ovarian fluid in endangered Caspian brown trout, *Salmo trutta caspius*, and its effects on spermatozoa motility and fertilizing ability compared to freshwater and a saline medium. *Fish Physiol. Biochem.* **2009**, *35*, 695–700. [CrossRef]
15. Łuczynski, M.J.; Nowosad, J.; Łuczynska, J.; Kucharczyk, D. Effect of Application of Different Activation Media on Fertilization and Embryo Survival of Northern Pike, (*Esox lucius*) under Hatchery Conditions. *Animals* **2022**, *12*, 1022. [CrossRef]
16. Rusco, G.; Di Iorio, M.; Iampietro, R.; Roncarati, A.; Esposito, S.; Iaffaldano, N. Cryobank of Mediterranean Brown Trout Semen: Evaluation of the Use of Frozen Semen up to Six Hours Post-Collection. *Fishes* **2021**, *6*, 26. [CrossRef]
17. Turner, E.; Montgomerie, R. Ovarian fluid enhances sperm movement in Arctic charr. *J. Fish Biol.* **2002**, *60*, 1570–1579. [CrossRef]
18. Urbach, D.; Folstad, I.; Rudolfsen, G. Effects of ovarian fluid on sperm velocity in Arctic charr (*Salvelinus alpinus*). *Behav. Ecol. Sociobiol.* **2005**, *57*, 438–444. [CrossRef]
19. Rosengrave, P.; Gemmell, N.J.; Metcalf, V.; McBride, K.; Montgomerie, R. A mechanism for cryptic female choice in chinook salmon. *Behav. Ecol.* **2008**, *19*, 1178–1185. [CrossRef]
20. Rosengrave, P.; Taylor, H.; Montgomerie, R.; Metcalf, V.; McBride, K.; Gemmell, N.J. Chemical composition of seminal and ovarian fluids of chinook salmon (*Oncorhynchus tshawytscha*) and their effects on sperm motility traits. *Comp. Biochem. Physiol. Part A* **2009**, *152*, 123–129. [CrossRef]
21. Rosengrave, P.; Montgomerie, R.; Gemmell, N. Cryptic female choice enhances fertilization success and embryo survival in chinook salmon. *Proc. R. Soc.* **2016**, *283*, 20160001. [CrossRef]
22. Dietrich, G.J.; Wojtczak, M.; Słowińska, M.; Dobosz, S.; Kuźmiński, H.; Ciereszko, A. Effects of ovarian fluid on motility characteristics of rainbow trout (*Oncorhynchus mykiss Walbaum*) spermatozoa. *J. Appl. Ichthyol.* **2008**, *24*, 503–507. [CrossRef]
23. Diogo, P.; Soares, F.; Dinis, M.T.; Cabrita, E. The influence of ovarian fluid on *Solea senegalensis* sperm motility. *J. Appl. Ichthyol.* **2010**, *26*, 690–695. [CrossRef]
24. Butts, I.A.E.; Johnson, K.; Wilson, C.C.; Pitcher, T.E. Ovarian fluid enhances sperm velocity based on relatedness in lake trout, *Salvelinus namaycush*. *Theriogenology* **2012**, *78*, 2105–2109. [CrossRef] [PubMed]
25. Butts, I.A.E.; Prokopchuk, G.; Kašpar, V.; Cosson, J.; Pitcher, T.E. Ovarian fluid impacts flagellar

beating and biomechanical metrics of sperm between alternative reproductive tactics. *J. Exp. Biol.* **2017**, *220*, 2210–2217. [CrossRef]
26. Galvano, P.M.; Johnson, K.; Wilson, C.C.; Pitcher, T.E.; Butts, I.A. Ovarian fluid influences sperm performance in lake trout, *Salvelinus namaycush*. *Reprod. Biol.* **2013**, *13*, 172–175. [CrossRef]
27. Alonzo, S.H.; Stiver, K.A.; Marsh-Rollo, S.E. Ovarian fluid allows directional cryptic female choice despite external fertilization. *Nat. Commun.* **2016**, *16*, 12452. [CrossRef]
28. Zadmajid, V.; Myers, J.N.; Sørensen, S.R.; Butts, I.A.E. Ovarian fluid and its impacts on spermatozoa performance in fish: A Review. *Theriogenology* **2019**, *132*, 144–152. [CrossRef]
29. Poli, F.; Immler, S.; Gasparini, C. Effects of ovarian fluid on sperm traits and its implications for cryptic female choice in zebrafish. *Behav. Ecol.* **2019**, *30*, 1298–1305. [CrossRef]
30. Purchase, C.F.; Rooke, A.C. Freezing ovarian fluid does not alter how it affects fish sperm swimming performance: Creating a cryptic female choice 'spice rack' for use in split-ejaculate experimentation. *J. Fish Biol.* **2020**, *96*, 693–699. [CrossRef]
31. Wilson-Leedy, J.G.; Ingermann, R.L. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. *Theriogenology* **2007**, *67*, 661–672. [CrossRef]
32. Cosson, J. Frenetic activation of fish spermatozoa flagella entails short-term motility, portending their precocious decadence. *J. Fish Biol.* **2010**, *76*, 240–279. [CrossRef]
33. Dietrich, G.J.; Kowalski, R.; Wojtczak, M.; Dobosz, S.; Goryczko, K.; Ciereszko, A. Motility parameters of rainbow trout (*Oncorhynchus mykiss*) spermatozoa in relation to sequential collection of milt, time of post-mortem storage and anesthesia. *Fish Physiol. Biochem.* **2005**, *31*, 1–9. [CrossRef]
34. Wojtczak, M.; Dietrich, G.J.; Słowińska, M.; Dobosz, S.; Kuźmiński, H.; Ciereszko, A. Ovarian fluid pH enhances motility parameters of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. *Aquaculture* **2007**, *270*, 259–264. [CrossRef]
35. Beirão, J.; Purchase, C.F.; Wringe, B.F.; Fleming, I.A. Wild Atlantic cod sperm motility is negatively affected by ovarian fluid of farmed females. *Aquacult. Environ. Interact.* **2014**, *5*, 61–70. [CrossRef]
36. İnanan, B.E.; Öğretmen, F. Determination of differences in the biochemical properties of sperm activating and non-activating ovarian fluids and their influences on sperm motility in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **2015**, *448*, 539–544. [CrossRef]
37. Hamano, S. On the spermatozoa agglutinating agents of the dog salmon and rainbow trout eggs. *Bull. Jpn. Soc. Sci. Fish.* **1961**, *27*, 225–231. [CrossRef]
38. Nomura, M. Studies on reproduction of rainbow trout, *Salmo gairdneri* with special reference to egg taking. VI. The activities of spermatozoa in different diluents and preservation of semen. *Bull. Jpn. Soc. Sci. Fish.* **1964**, *30*, 723–733. [CrossRef]
39. Billard, R. A new technique of artificial insemination for salmonids using a sperm diluent. *Fisheries* **1977**, *2*, 24–25.

40. Aegerter, S.; Jalabert, B. Effects of post-ovulatory oocyte ageing and temperature on egg quality and on the occurrence of triploid fry in rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* **2004**, *231*, 59–71. [CrossRef]
41. Billard, R.; Petit, J.; Jalabert, B.; Szollosi, D. Artificial insemination in trout using a sperm diluant. In *The Early Life History of Fish*;
Blaxter, J.H.S., Ed.; Springer: Berlin/Heidelberg, Germany, 1974; pp. 715–723.
42. Billard, R. Effects of coelomic and seminal fluids and various saline diluents on the fertilizing ability of spermatozoa in the rainbow trout, *Salmo gairdneri*. *J. Reprod. Fert.* **1983**, *68*, 77–84. [CrossRef]
43. Billard, R.; Cosson, M.P. Sperm motility in rainbow trout *Parasalmo mykiss*: Effect of pH and temperature. *Reprod. Biol. Endocrinol.* **1988**, 161–167.
44. Lahnsteiner, F.; Weismann, T.; Patzner, R. Composition of the ovarian fluid in 4 salmonid species: *Oncorhynchus mykiss*, *Salmo trutta f lacustris*, *Saivelinus alpinus* and *Hucho hucho*. *Reprod. Nutr. Dev.* **1995**, *35*, 465–474. [CrossRef] [PubMed]
45. Kholodnyy, V.; Gadêlha, H.; Cosson, J.; Boryshpolets, S. How do freshwater fish sperm find the egg? The physicochemical factors guiding the gamete encounters of externally fertilizing freshwater fish. *Rev. Aquac.* **2020**, *12*, 1165–1192. [CrossRef]
46. Gage, M.J.; Macfarlane, C.P.; Yeates, S.; Ward, R.G.; Searle, J.B.; Parker, G.A. Spermatozoal traits and sperm competition in Atlantic salmon: Relative sperm velocity is the primary determinant of fertilization success. *Curr. Biol.* **2004**, *14*, 44–47. [CrossRef]
47. Figueroa, E.; Lee-Estevez, M.; Valdebenito, I.; Watanabe, I.; Oliveira, R.P.S.; Romero, J.; Castillo, R.L.; Farías, J.G. Effects of cryopreservation on mitochondrial function and sperm quality in fish. *Aquaculture* **2019**, *511*, 634190. [CrossRef]
48. Lorenzoni, M.; Carosi, A.; Giovannotti, M.; La Porta, G.; Splendiani, A.; Barucchi, V.C. Ecology and conservation of the Mediterranean trout in the central Apennines (Italy). *J. Limnol.* **2019**, *78*, 1. [CrossRef]
49. D'Agaro, E.; Gibertoni, P.; Marroni, F.; Messina, M.; Tibaldi, E.; Esposito, S. Genetic and Phenotypic Characteristics of the *Salmo trutta* Complex in Italy. *Appl. Sci.* **2022**, *12*, 3219. [CrossRef]
50. Polgar, G.; Iaia, M.; Righi, T.; Volta, P. The Italian Alpine and Subalpine trouts: Taxonomy, Evolution, and Conservation. *Biology* **2022**, *11*, 576. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

STUDY 2

Fishes (2023)
(doi.org/10.3390/fishes8040190)

fishes

MDPI

Brief Report

Zootechnical Brown Trout (*Salmo trutta* L. 1758) Ovarian Fluid Fails to Upregulate the Swimming Performances of Native Mediterranean Brown Trout (*Salmo cettii* Rafinesque, 1810) Sperm in the Biferno River

Giusy Rusco [1], Michele Di Iorio [1], Stefano Esposito [2,*], Pierpaolo Gibertoni [2], Emanuele Antenucci [1], Valentino Palombo [1], Alessandra Roncarati [3] and Nicolaia Iaffaldano [1,*]

[1] Department of Agricultural, Environmental and Food Sciences, University of Molise, Via De Sanctis snc, 86100 Campobasso, Italy; giusy.rusco@unimol.it (G.R.); michele.diiorio@unimol.it (M.D.I.); e.antenucci@studenti.unimol.it (E.A.); valentino.palombo@unimol.it (V.P.)
[2] Mediterranean Trout Research Group "MTRG", 42032 Ventasso, Italy; pierpaologibertoni72@gmail.com
[3] School of Biosciences and Veterinary Medicine, University of Camerino, Viale Circonvallazione 93–95, 62024 Matelica, Italy; alessandra.roncarati@unicam.it
* Correspondence: dott.stefanoesposito@gmail.com (S.E.); nicolaia@unimol.it (N.I.); Tel.: +39-0874-404697 (N.I.); Fax: +39-0874-404855 (N.I.)

Abstract: In external fertilizer fish, ovarian fluid (OF) seems to play a key role in fertilization success, improving spermatozoa swimming performance. These OF/sperm interaction mechanisms are frequently species-specific and/or population-specific and could decrease the risk of genetic introgression of wild populations from introduced or escaped zootechnical individuals. The Mediterranean brown trout (*Salmo cettii*) is threatened by genetic introgression with strains of domestic brown trout (*Salmo trutta*) that were introduced for recreational purposes. The aim of our study was to test if native *S. cettii* females, rather than zootechnical S. *trutta*, produce OF with a greater ability to upregulate the sperm motility of conspecific males. Thus, we compared the sperm swimming performances of males inhabiting the Biferno River (Molise region—Southern Italy) activated in native *S. cettii* vs. zootechnical *S. trutta* female's OFs. In our study, native females' OFs (20% diluted), compared to spring water, has the ability to significantly boost the sperm performance of the autochthonous males, while zootechnical *S. trutta* fails. These preliminary results suggest that OF-sperm interactions could potentially influence or direct the hybridization mechanisms involving the native Mediterranean trout inhabiting the Biferno River and the domestic lineage of brown trout introduced in the past.

Keywords: reproductive biology; local adaptation; sperm motility; hostile reproductive environment

Key Contribution: During natural reproduction, the aqueous environment becomes hostile, decreasing the chances of fertilization success. Ovarian fluid promotes close contact between gametes, creating a stabilized fertilization microenvironment. Ovarian fluids of native *S. cettii* females improve sperm swimming performance of conspecific males compared to spring water, whilst ovarian fluids of zootechnical *S. trutta* fail.

check for updates

Citation: Rusco, G.; Di Iorio, M.; Esposito, S.; Gibertoni, P.; Antenucci, E.; Palombo, V.; Roncarati, A.; Iaffaldano, N. Zootechnical Brown Trout (*Salmo trutta* L. 1758) Ovarian Fluid Fails to Upregulate the Swimming Performances of Native Mediterranean Brown Trout (*Salmo cettii* Rafinesque, 1810) Sperm in the Biferno River. *Fishes* **2023**, *8*, 190. https://doi.org/10.3390/fishes8040190

Academic Editor: Shi-Jian Fu

Received: 9 February 2023
Revised: 29 March 2023
Accepted: 30 March 2023
Published: 31 March 2023

Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1.Introduction

During natural reproduction, a common feature of all externally fertilizing freshwater fish is that they expel their gametes into the aqueous environment. Although the sperms need the water to activate their motility (Morisawa et al., 1985), the aqueous environment is hostile and decreases the chances of fertilization success, due to its peculiar physical (turbulent flow, temperature variation) and chemical properties (Billard et al.,1981; Petersen et al.,2001; Hoysak et al., 2001; Liao et al., 2018; Kholodnyy et al., 2020). Thus, specific mechanisms guiding sperm may have evolved to maximize the sperm–egg encounter under extreme environmental conditions (Kholodnyy et al., 2020; Lahnsteiner al., 2002; Elofsson al., 2006; Lehnert al., 2017). In particular, after sperm ejaculation, OF promotes close contact between gametes, reducing their dispersion and creating a stabilized fertilization microenvironment (Lahnsteiner et al., 2002; Rosengrave et al., 2009). Several studies have shown that ovarian fluid could significantly affect the swimming performances of sperm (Rosengrave et al., 2009; Turner et al., 2002; Urbach et al., 2005; Dietrich et al., 2008; Diogo et al., 2010; Galvano et al., 2013; Butts et al., 2012,2017; Poli et al., 2019; Rosengrave et al., 2008; Beirão et al., 2014) and could consequently influence the outcome of fertilization in terms of fertilized oocytes rate in different fish species (Chinook salmon, Pacific herring, Caspian brown trout, turbot, ocellated wrasse) (Lahnsteiner et al., 2002; Rosengrave et al., 2016; Cherr et al., 2008; Hatef et al., 2009; Jia et al.,2015; Alonzo et al., 2016;)

According to some authors, these ovarian fluid–sperm interaction mechanisms could be species-specific and population-specific (Yeates et al.,2013; Beirão et al.,2015; Zadmajid et al.,2019). In addition to parental genotypes, the interactions can be influenced by environmental factors (i.e., density and physical–chemical water parameters) and dietary factors, as stated by Beirão et al.2014. These authors showed that sperm from wild males of Atlantic Cod was negatively affected by ovarian fluids of farmed females, suggesting possible relation to nutritional deficiencies of farmed individuals. Therefore, these phenomena could potentially play an important role in rivers inhabited by native salmonids where interspecific hybridization with allochthonous species/or strains represents a main threat to local populations.

The Mediterranean brown trout (*Salmo cettii*[1]) is an endemic freshwater species of the Mediterranean area whose conservation status is currently considered “near threatened” at the European level and “critical endangered” in Italy, according to IUCN Red Lists, mainly due to habitat degradation (dam building, river straightening, local pollution) and to the genetic introgression with zootechnical species introduced for recreational purposes. The introduction of *S. trutta* began at the end of the 19th century and had a severe impact on native populations inhabiting the Italian Peninsula and the main Mediterranean Islands (Splendiani et al., 2016, 2017; Berrebi 2015; Sabatini et al., 2011;). This

phenomenon has led to the introgression of domestic genes into the native genome and, in many cases, to its complete replacement. Indeed, Splendiani et al. reported that about 60% of the native Apennine populations are severely introgressed or have been completely replaced by S. trutta, and only 10% are pure or show an introgression degree lower than 0.10 (Splendiani et al., 2016).

In particular, native populations of Mediterranean brown trout inhabiting the Biferno and Volturno river basins (Molise region—Southern Italy) are currently the target of the conservation project LIFE Nat. Sal. Mo. This project aims to recover the genetic integrity of wild *S. cettii* populations through the creation of the first semen cryobank of Mediterranean trout in Europe, which is used to maximize the genetic variability of the offspring in a supportive breeding program (Di Iorio et al., 2019,2023; Rusco et al., 2019,2020,2021; Ferguson et al., 2019). The populations of the main courses of Molise, characterized by a distinctive migratory tactic, were the least introgressed of the entire project area despite the massive introduction of zootechnical Salmo trutta that occurred throughout the past decades (Palombo et al., 2021). We hypothesized that this protective effect is mainly due to the peculiar migation patterns of the native population and to adaptive selection. Similarly, Jurlina et al. 2020 studied Adriatic streams of the Western Balkans and observed that migratory tactics and life-history plasticity preserves the original genetic structure of locally adapted populations, suggesting that these features act as a stabilizing population mechanism that protects them from introgression by allochthonous *S. trutta* strains.

Furthermore, in the study area, Palombo et al., 2021 found discordance between nuclear (LDH-C1*) and mitochondrial (16 s) markers, showing the frequencies of foreign (maternal) mtDNA locus to be lower than expected. These anomalies are frequently driven by sex-biased asymmetries and selective introgression or by genetic drift on mtDNA Toews et al., 2012. Considering the massive supplementation of domestic strains carrying continuously allochthonous matrilinear haplotypes, we can speculate about the higher reproductive success of native females rather than the introduced ones. In light of these considerations and previous research (Beirão, et al., 2014,2015; Yeates et al., 2013; Zadmajid et al.,2019), the rationale of this study is to evaluate the potential ability of native *S. cettii* rather than zootechnical *S. trutta* ovarian fluids to upregulate the sperm motility parameters of native males, contributing to a potential increased fertilization chances in a flowing and turbulent environment for native females.

[1]Mediterranean brown trout is listed in Annex II of the Habitat Directive under the taxon Salmo macrostigma, but is currently reported as *Salmo cettii* in the Italian and European Red Lists and in the Member States' conservation status assessments of the "habitats types and species of Community interest" (Art. 17). Recent genetic evidence suggests that Italian peninsular Mediterranean brown trout belong to a separate taxon named *S. ghigii*, limiting the name *S. cettii* to Sicilian trout (Lorenzoni et al.,2019; D′Agaro et al., 2022; Polgar et al., 2022). Although we agree with the recent observations, we still use the name *S. cettii* in the current paper, because

Mediterranean brown trout populations are still protected by the Habitat Directive and subsequent conservation status updates under this taxon.

2. Materials and Methods

2.1. Animals

Breeders from the native Mediterranean brown trout (*Salmo cettii*) wild population were captured in the Biferno River (Molise region, Southern Italy—Adriatic basin) during the spawning season (January–February 2022) using electro-fishing and fixed traps. The fish were captured within LIFE Nat.Sal.Mo Project's activities (LIFE17 NAT/IT/000547). The sampling sites coincide with a spawning area located close to the springs of the Biferno River at Bojano (CB) frequented by a native migrant population characterized by a mean introgression rate lower than 10%. We collected semen samples and OFs used in the experiment from ten wild males and five wild-tagged females genetically characterized as "native individuals" by Palombo et al. 2021 (Native admixture ancestry $q_i > 0.95$). The males belonged to the 2+ and 3+ classes, with an average total length of 31.9 ± 3.2 cm (range = 2.5 - 36.0 cm). The females were 3+ and 5+ years-old and characterized by an average total length of 45.1 ± 4.7 cm (range = 39.0 - 53.5 cm).

In addition, OFs were collected from five zootechnical brown trout (*S. trutta*) females reared in a commercial hatchery that breeds domestic strain of *S. trutta*. Zootechnical *S. trutta* females were reared at a growth rate similar to that of wild Mediterranean trout of the Biferno River used in the experiment. *S. trutta* females were 4+ years-old, with an average total length of 50.2 ± 1.7 cm (range = 48.0–53.0 cm). No brood stock was sedated prior to sampling eggs or semen.

2.2. Ovarian Fluid and Sperm Collection and Analytical Measurements

Eggs were collected by an abdominal massage from five native *S. cettii* and five zootechnical S. trutta females. OF was separated from each egg batch directly with a syringe after egg decantation. After collection, each individual OF sample was subjected to the pH measurement using a BasiC 20 pH-meter (CRISON instruments, Barcelona, Spain). Then, OF samples were frozen because of the impossibility to collect all the sperm and ovarian fluid samples on the same day (Purchase et al., 2020). Moreover, OF samples were individually stored in at least five 1.5 mL microtubes in order to avoid freeze–thaw cycles.

In February, during the peak of the natural spawning season, semen samples were collected from ten native males by abdominal massage after careful cleaning of the urogenital papilla. Semen was stored on ice, transferred from the river to the laboratory and used within 4 h of collection. Sperm concentration was measured using a photometric method. Briefly, the optical density of the diluted semen in 0.9% NaCl with a ratio of 1:200 (*v/v*) was measured using a portable photometer DR 1900

(HACH Company, Loveland, CO, USA) at a wavelength of 530 nm. The value of the sperm concentration was extrapolated using a standard curve established previously by relating the optical density with the sperm concentration expressed as $\times 10^9$ sperm/mL, following the procedure described by Nynca and Ciereszko 2009. Sperm viability was measured using the Muse® Cell Analyzer flow cytometer (Luminex corporation, 12212 Technology Blvd Suite 130, Austin, TX, USA) according to the manufacturer's protocol. Semen samples were diluted in PBS to obtain concentrations in the range of 1×10^5 to 1×10^7 spermatozoa/mL. Subsequently, 20 µL of the diluted semen was mixed with 780 µL (dilution factor 1:40) of Muse Count and Viability Reagent in an Eppendorf tube (Luminex corporation 12212 Technology Blvd Suite 130, Austin, TX, USA), then incubated for 5 min at room temperature and analysed with the flow cytometry.

Nucleated cells were stained using a membrane-permeant DNA-staining dye in order to differentiate cells with a nucleus from debris and non-nucleated cells. A DNA binding Muse dye based on 7-aminoactinomycin D (7-AAD) stains cells that have lost their membrane integrity, allowing the dye to stain the nucleus of dead and dying cells. This parameter differentiates viable (live cells that do not stain) from non-viable (dead or dying cells that stain) cells. Sperm pH was determined by an electrode using a Basic 20 pH-meter.

2.3. Experimental Design

Each semen sample (N = 10) was analysed individually in the presence of OFs from five native *S. cettii* (NOF) and five zootechnical *S. trutta* females (ZOF) in a full factorial design (10×10). OF samples were diluted at 20% (*v/v*) in spring water (SW; pH = 8.1). SW was sampled at a source of the Biferno River near the spawning sites. The sperm are likely to encounter different OF concentrations when moving towards the eggs in natural environments. The OF dilution in the surrounding environment determines the physical and chemical properties of the activation media that could affect the behaviour of the sperm (Zadmajid et al., 2019). Many authors, for salmonids, have used dilution ranging from 10 to 100% OF (Turner and Montgomerie et al., 2002; Rosengrave et al., 2008, 2016; Alonzo et al., 2016; Purchase et al., 2020). The choice of a 20% dilution rate was in accordance with Butts et al. 2012, which suggests that this OF dilution could represent the concentrations of OF during a natural spawning event of lake trout. Purchase and Rooke 2020 also used a dilution with an OF water ratio of 1:4 (20%), testing the utilization of frozen ovarian fluid to assess the OFs effect on sperm swimming performance of lake trout, brown trout and Atlantic salmon. Sperm motility parameters recorded in 20%NOF and 20%ZOF (hereafter simply referred to as NOF and ZOF) were compared with each other and with that obtained in SW alone in order to reveal the changes that occur in swimming patterns when spermatozoa are released into the water and move to

the eggs of both species. A saline activation medium (AM; pH = 8.85) consisting of 1 mM CaCl2, 20 mM Tris, 30 mM glycine and 125 mM NaCl, at pH 9.0 (Billard 1992), supplemented with 0.5% bovine serum albumin, was used as a control solution, in order check if the semen samples were suitable for their use in the experiment on the basis of their initial quality The experiment was conducted on five different days over the period of two weeks, dividing each day into two work sessions (morning and afternoon), one for each semen sample, collected respectively in the early morning and early afternoon (5 days × 2 males). Each day one microtube of OF from each female was thawed at 4°C for 2 h and then kept on ice for the duration of the analysis.

2.4. Sperm Motility Analysis

Sperm motility analysis was performed using a Computer-Assisted Sperm Analysis (CASA) system coupled with a phase contrast microscope (Nikon model Ci-L negative contrast, Firenze, Italy) employing the Sperm Class Analyzer (SCA) software (VET Edition, Barcelona, Spain). An aliquot of 1 µL of semen from each male was activated in 300 µL of OF from each female in a randomized order, SW or AM (activation solutions), carrying out two replicate sperm-activations for each treatment combination (10 males × 12 activation solutions × 2 sperm-activations replicates = 240 observations). After rapid mixing, 0.7 µL of each dilution was immediately placed into a well (diameter 5 mm) of a 12-well multi test glass slide (TEKDON Inc., Myakka City, FL, USA) and covered with a coverslip. Sperm motility parameters were analysed 10 s post-activation using a 25 fps rate during recording. The following sperm traits were evaluated: motile spermatozoa (MOT, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), straightness (STR, %), beat cross frequency BCF, Hz) and amplitude of lateral displacement of the spermatozoon head (ALH, µm).

The duration of sperm movement (DSM) was chronometer and calculated as the time from sperm activation until movement cessation of spermatozoa in the field of view. We consider spermatozoa vibration as a parameter to establish the cessation of movement.

The measurements took place in a lab with temperatures set at 17–18°C. The loading of chambers and the recording of motility were both carried out by the same operator, taking care to standardize the operation timing from ice to observation. Although higher room temperature compared to the natural environment (10–12°C) may have reasonably affected the duration of motility and velocity of spermatozoa, as reviewed by Dadras et al. 2016. This bias will have affected all measurements equally.

2.5. Statistical Analysis

All statistical analyses were performed using the statistical software R (Version 4.2.0, R Core Team, Vienna, Austraia), at significance levels of $p \leq 0.05$. Replicates of each cross were treated as repeated measures, using its mean for statistical analysis to meet the independency of observations assumption. MOT (%) measurements were arcsine transformed prior to analysis.

The differences among treatments for all tested sperm motility parameters were analysed using Mixed Model ANOVAs with the activation medium as a fixed factor and the male's identity (ID) as a random factor, followed by Tukey's post hoc test for multiple comparisons between the groups (*lme4*, *lmerTest* and *multcomp* packages). The AIC test was used to compare different models (with or without males' random effect) and determine which one was the best fit for the data (*lmerTest* package). The outliers were detected using the boxplot()$out function. Normality and homoscedasticity were tested by visual inspection of the residuals' graphs, Shapiro–Wilk test and Levene's test (*rstatix* and *car* packages). After transformation for normality, the model with MOT showed no homogeneity of variance (Levene's test: df = 2, F = 13.7, $p < 0.05$). Then, MOT was analysed through GLMMs setting the Gaussian family (*lme4* package).

Three-way ANOVAs were conducted, setting as fixed factors NOF vs. ZOF, males ID and females ID, to test the main effect of the males and females used in the experiment (*stats* package).

To check if the individual addictive effect of males in OFs correlated with the VCL's control values registered in AM, we tested the correlation using the cor.test() function, setting the Pearson's method. The graphics were generated by the *ggplot2* package.

3. Results

3.1. Analytical Measurements of Semen and Ovarian Fluid

All sperm motility parameters recorded in AM to check the sperm's initial quality are reported in the supplementary material (Table S1). The average sperm concentration and viability were 15.68 ± 7.19 10^9 sperm/mL and 94.69 ± 2.67% respectively. The average semen pH measured was 7.91 ± 0.19, while for native and zootechnical OFs it was 8.36 ± 0.09 and 8.56 ± 0.19, respectively. The pH values of each semen and OF sample were reported in the supplementary material (Table S2).

3.2. Comparison between Spring Water, ZOF and NOF as Activation Media

The random effect of males explained a significant proportion of variance for al tested parameters. Then, using Mixed Models with males' random effect, we registered a significant increment of MOT, VCL, VAP, VSL and DSM in NOF compared to SW alone. In contrast, ZOF failed to significantly boost the swimming performances for the motility parameters reported above (Figure 1). For STR, LIN and ALH, we found significant differences among all groups. No significant difference between NOF and ZOF was found for BCF.

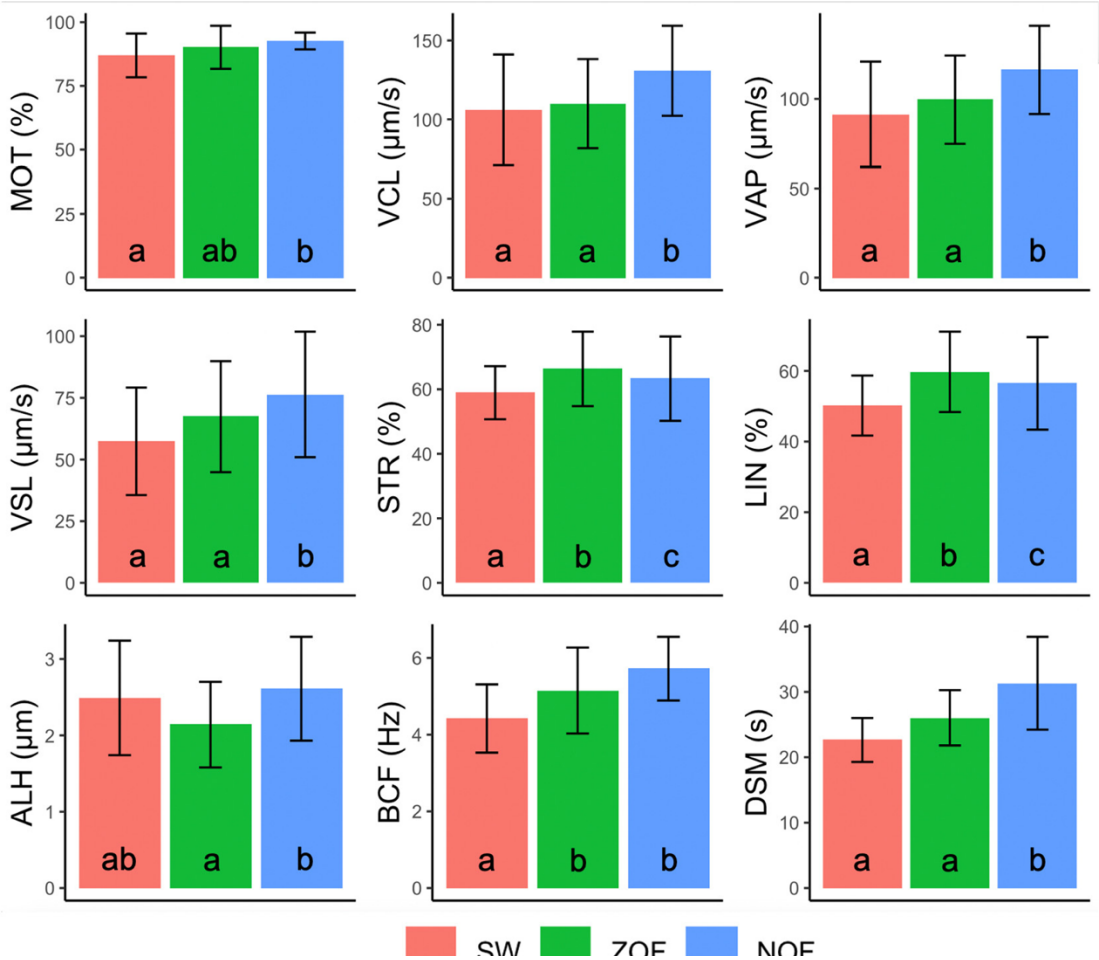


Figure 1. Barplots displaying the variability among treatments (spring water—SW; 20% native ovarian fluid—NOF; 20% zootechnical ovarian fluid—ZOF) for each sperm motility parameter, presented as Means ± SD (observations number in: SW= 10; NOF = 50; ZOF = 50). Error bars represent standard deviation. Different letters show significant differences among treatments.

3.3. Exploring the Effects of Specific Males and Females Used in the Experiment

The three-way ANOVAs show significant differences between NOF and ZOF for each kinetic parameter (Table 1). All motility parameters except STR and LIN are higher ($p < 0.05$) in NOF than in ZOF. Further, the analysis shows a significant male effect on all sperm motility parameters, while the female effect was significant only for VCL, VAP and STR (Table 1).

The individual addictive effect of males—and the related inter-male variability—was correlated to the values registered with activation in AM (Figure 2), in accordance with the use of our activation medium as a proxy for the evaluation of semen quality. According to these findings, inter-male variability and female origin represent the main effects that explain most of the observed variability in our data.

Table 1. Significant levels of the fixed effects of male ID, female ID and the effect of belonging of female to the native or zootechnical group (N-Z group) for all sperm traits.

	Male ID	**Female ID**	**N-Z Group**
Sperm Trait	**Significance**	**Significance**	**Significance**
MOT (%)	***		*
VCL (µm/s)	***	**	***
VAP (µm/s)	***	**	***
VSL (µm/s)	***		***
STR (%)	***	*	**
LIN (%)	***		**
ALH (µm)	***		***
BCF (Hz)	***		*
DSM (s)	***		***

Significant levels: $p < 0.05$ *, $p < 0.01$ ** and $p < 0.001$ ***

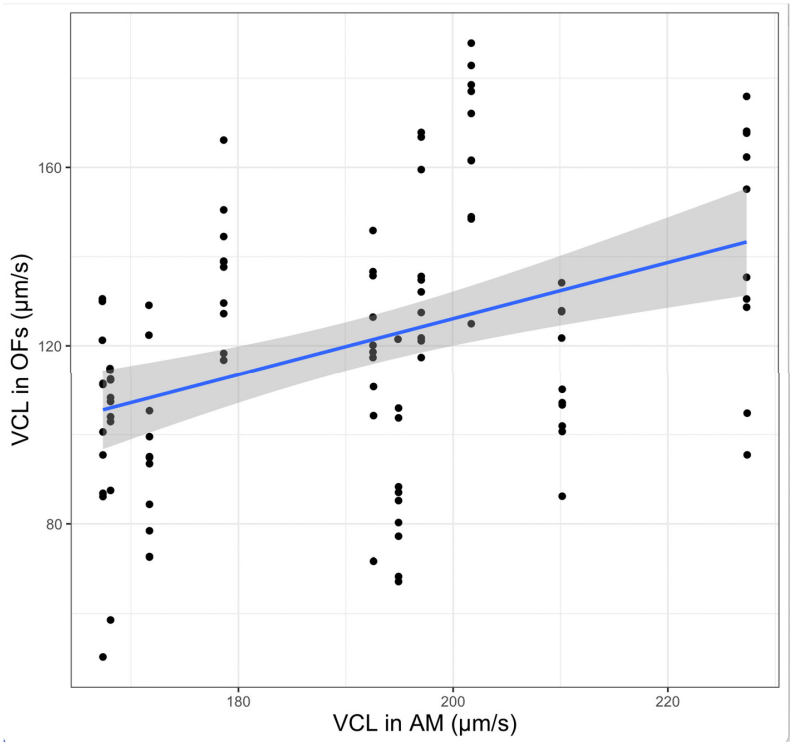


Figure 2. The VCL (curvilinear velocity) values registered in all OFs (native and zootechnical) are significantly correlated to those registered in AM ($p < 0.05$). The blue line represents the fitted linear regression and the dark shadow shows the 95% confidence interval band.

4. Discussion

In this study, we showed that ovarian fluids of native S. cettii females have a significant enhancing effect on sperm swimming parameters of native wild males compared to SW alone, while zootechnical *S. trutta* OFs fail.

Interestingly, the presence of NOF, rather than ZOF, induced a significant increase of VCL, VAP, VSL and ALH parameters and of DSM (longevity) compared to SW. The variation in sperm performances observed in our study within each group (NOF and ZOF) was mainly explained by the inter-male variability effect (male ID). Consistent with other similar studies (Urbach et al., 2005; Dietrich et al., 2008; Galvano et al., 2013; Rosengrave et al., 2008) some males produce sperm with intrinsically superior motility features than others, resulting in better performances overall in the OFs. On the other hand, the effect of female ID was significant only for some parameters, such as VCL, VAP and STR, suggesting that some females produce OF with a higher capability to increase the sperm traits of males.

The differences that were observed in sperm motility patterns between groups NOF and ZOF could affect the fertilization success in relation to the female origin. It is known that, in fish with external fertilization, the sperm reproductive success could be strongly influenced by hostile environmental conditions, i.e., turbulent aqueous medium (Petersen et al., 1992, 1998, 2001) and by the spermatozoa's ability to rapidly find the micropyle within the short time available (Kima et al.,2001). The VCL seems to be an essential prerequisite for sperm to rapidly swim around the egg to find the micropylar canal and fertilize the eggs (Cherr et al., 2008; Kime et al., 2001; Yanagimachi et al., 2017). Moreover, several authors reported the existence of a positive correlation between fertilization rate and some sperm motility parameters (VCL and longevity) in fish, including salmonids (Beirão et al., 2015; Kime et al., 2001; Lahnsteiner et al.,1998; Gage et al., 2004; Linhart et al., 2005). The native ovarian fluid ability to upregulate the curvilinear velocity and the duration of progressive movement could promote the encounter between gametes in such unfavourable environments, thus increasing the fertilization rate. The zootechnical *S. trutta* OFs inability to increase the swimming performance of sperm compared to SWcould lead to less efficient fertilization of the deposed eggs batch, potentially reducing the number of hybrid offspring. In conclusion, our results suggest that the native *S. cettii* females, rather than zootechnical S. trutta, have a superior ability to boost the sperm motility of Mediterranean trout males, as a potential fine-adapted mechanism to favour the egg-sperm encounter. However, to fully confirm the fertilization outcomes, it would be necessary to test the in vivo fertilization, reproducing the same critical natural conditions as the flowing water, turbulences and gamete's expulsion dynamics. Conventional fertilization trials could easily lead to erroneous conclusions, suggesting that the static fertilization environment of artificial reproduction is very

different from the challenging, natural one. Furthermore, a full-factorial experimental design involving 10 females × 10 males × 2 replicates scheme (n. of observations = 200) allowed us to make our main result robust, but it completely consumes the limited volumes of semen and OFs collected from the breeders involved in the experiment. Thus, the samples were not sufficient to conduct further analyses on semen and OFs composition. That could explain the found differences between ZOF and NOF regarding their boosting ability.

Therefore, in order to explore the nature of compatibility interaction between male and female gametes, we are planning to test how these interactions are affected by the chemicalphysical and biological parameters of both semen and OFs. Further ionic composition and proteomic analysis could help to resolve the nature of the gametes compatibility mechanisms observed in our study.

5. Conclusions

The main outcomes of this preliminary study showed that, compared to spring water, NOF enhanced the sperm swimming performance of native males, whilst ZOF failed. A potential ovarian fluid-driven recognition mechanism might have evolved to increase the fertilization success in native populations of Mediterranean brown trout. Further studies are planned to elucidate the causes behind it.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes8040190/s1, Table S1: Sperm motility parameters of Mediterranean trout (N = 10) activated in artificial medium (AM); Table S2: Seminal plasma and ovarian fluid pH measured for each male and female.

Author Contributions: Conceptualization, N.I., G.R. and S.E.; Data curation, N.I., S.E., M.D.I., V.P. and G.R.; Formal analysis, G.R., M.D.I., V.P. and S.E.; Methodology, G.R., M.D.I., P.G. and E.A.; Writing—original draft, G.R. and S.E.; Writing—review & editing, A.R., N.I. and M.D.I.; Funding Acquisition, N.I. and S.E. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the LIFE Nat.Sal.Mo. project (LIFE17 NAT/IT/000547).

Institutional Review Board Statement: The experiments were conducted in accordance with the Code of Ethics of the EU Directive 2010/63/EU for animal experiments. This study is part of a Nat.Sal. Mo LIFE project that received “a positive opinion” from the Ministry of the Environment and the Protection of the Territory and the Sea. The sampling and handling of fish followed animal welfare practices as reported in the Ministerial Protocol (ISPRA). All experiments were carried out with the appropriate authorizations from the Molise Region–Dipartimento Governo del Territorio, Mobilità e Risorse Naturali cod. DP.A4.02.4N.01 (protocol number 3969, 3 August 2018), according to the current regulations on the protection of the species, biosecurity, protocols of sampling of fresh water and animal welfare.

Data Availability Statement: Data sharing not applicable.

Acknowledgments: The authors thank Amber Burchell for the English language revision of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Morisawa, M. Initiation mechanism of sperm motility at spawning in teleosts. *Zool. Sci.* **1985**, *2*, 605–615.
2. Billard, R.; Bry, C.; Gillet, C. Stress, environment and reproduction in teleost fish. In *Stress and Fish*; Pickering, A.D., Ed.; Academic Press: London, UK, 1981; pp. 185–208.
3. Petersen, C.W.; Warner, R.R.; Shapiro, D.Y.; Marconato, A. Components of fertilization success in the bluehead wrasse, *Thalassoma bifasciatum*. *Behav. Ecol.* **2001**, *12*, 237–245. [CrossRef]
4. Hoysak, D.J.; Liley, N.R. Fertilization dynamics in sockeye salmon and a comparison of 450 sperm from alternative male phenotypes. *J. Fish Biol.* **2001**, *58*, 1286–1300. [CrossRef]
5. Liao, W.B.; Huang, Y.; Zeng, Y.; Zhong, M.J.; Luo, Y.; Lüpold, S. Ejaculate evolution in external fertilizers: Influenced by sperm competition or sperm limitation? *Evolution* **2018**, *72*, 4–17. [CrossRef] [PubMed]
6. Kholodnyy, V.; Gadêlha, H.; Cosson, J.; Boryshpolets, S. How do freshwater fish sperm find the egg? The physicochemical factors guiding the gamete encounters of externally fertilizing freshwater fish. *Rev. Aquac.* **2020**, *12*, 1165–1192. [CrossRef]
7. Lahnsteiner, F. The influence of ovarian fluid on the gamete physiology in the Salmonidae. *Fish Physiol. Biochem.* **2002**, *27*, 49–59. [CrossRef]
8. Elofsson, H.; Van Look, K.J.W.; Sundell, K.; Sundh, H.; Borg, B. Stickleback sperm saved by salt in ovarian fluid. *J. Exp. Biol.* **2006**, *209*, 4230–4237. [CrossRef]
9. Lehnert, S.J.; Butts, I.A.E.; Flannery, E.W.; Peters, K.M.; Heath, D.D.; Pitcher, T.E. Effects of ovarian fluid and genetic differences on sperm performance and fertilization success of alternative reproductive tactics in Chinook salmon. *J. Evol. Biol.* **2017**, *30*, 1236–1245. [CrossRef]
10. Rosengrave, P.; Taylor, H.; Montgomerie, R.; Metcalf, V.; McBride, K.; Gemmell, N.J. Chemical composition of seminal and ovarian fluids of chinook salmon (*Oncorhynchus tshawytscha*) and their effects on sperm motility traits. *Comp. Biochem. Physiol.* **2009**, *152*, 123–129. [CrossRef]
11. Turner, E.; Montgomerie, R. Ovarian fluid enhances sperm movement in Arctic charr. *J. Fish Biol.* **2002**, *60*, 1570–1579. [CrossRef]
12. Urbach, D.; Folstad, I.; Rudolfsen, G. Effects of ovarian fluid on sperm velocity in Arctic charr (*Salvelinus alpinus*). *Behav. Ecol. Sociobiol.* **2005**, *57*, 438–444. [CrossRef]
13. Dietrich, G.J.; Wojtczak, M.; Słowińska, M.; Dobosz, S.; Kuźmiński, H.; Ciereszko, A. Effects of ovarian fluid on motility characteristics of rainbow trout (*Oncorhynchus mykiss Walbaum*) spermatozoa. *J. Appl. Ichthyol.* **2008**, *24*, 503–507. [CrossRef]
14. Diogo, P.; Soares, F.; Dinis, M.T.; Cabrita, E. The influence of ovarian fluid on Solea senegalensis sperm motility. *J. Appl. Ichthyol.* **2010**, *26*, 690–695. [CrossRef]
15. Galvano, P.M.; Johnson, K.; Wilson, C.C.; Pitcher, T.E.; Butts, I.A. Ovarian fluid influences sperm performance in lake trout, *Salvelinus namaycush*. *Reprod. Biol.* **2013**, *13*, 172–175. [CrossRef]
16. Butts, I.A.E.; Johnson, K.; Wilson, C.C.; Pitcher, T.E. Ovarian fluid enhances sperm velocity based

on relatedness in lake trout, *Salvelinus namaycush*. *Theriogenology* **2012**, *78*, 2105–2109. [CrossRef]
17. Butts, I.A.E.; Prokopchuk, G.; Kašpar, V.; Cosson, J.; Pitcher, T.E. Ovarian fluid impacts flagellar beating and biomechanical metrics of sperm between alternative reproductive tactics. *J. Exp. Biol.* **2017**, *220*, 2210–2217. [CrossRef]
18. Poli, F.; Immler, S.; Gasparini, C. Effects of ovarian fluid on sperm traits and its implications for cryptic female choice in zebrafish. *Behav. Ecol.* **2019**, *30*, 1298–1305. [CrossRef]
19. Rosengrave, P.; Gemmell, N.J.; Metcalf, V.; McBride, K.; Montgomerie, R. A mechanism for cryptic female choice in chinook salmon. *Behav. Ecol.* **2008**, *19*, 1178–1185. [CrossRef]
20. Beirão, J.; Purchase, C.F.; Wringe, B.F.; Fleming, I.A. Wild Atlantic cod sperm motility is negatively affected by ovarian fluid of farmed females. *Aquac. Environ. Interact.* **2014**, *5*, 61–70. [CrossRef]
21. Rosengrave, P.; Montgomerie, R.; Gemmell, N. Cryptic female choice enhances fertilization success and embryo survival in chinook salmon. *Proc. R. Soc. B* **2016**, *283*, 20160001. [CrossRef]
22. Cherr, G.N.; Morisawa, M.; Vines, C.A.; Yoshida, K.; Smith, E.H.; Matsubara, T.; Pillai, M.C.; Griffin, F.J.; Yanagimachi, R. Two egg-derived molecules in sperm motility initiation and fertilization in the Pacific herring (*Clupea pallasi*). *Int. J. Dev. Biol.* **2008**, *52*, 743–752. [CrossRef] [PubMed]
23. Hatef, A.; Niksirat, H.; Alavi, S.M. Composition of ovarian fluid in endangered Caspian brown trout, *Salmo trutta caspius*, and its effects on spermatozoa motility and fertilizing ability compared to freshwater and a saline medium. *Fish Physiol. Biochem.* **2009**, *35*, 695–700. [CrossRef] [PubMed]
24. Jia, Y.D.; Niu, H.X.; Meng, Z.; Liu, X.F.; Lei, J.L. Biochemical composition of the ovarian fluid and its effects on the fertilization capacity of turbot *Scophthalmus maximus* during the spawning season. *J. Fish Biol.* **2015**, *86*, 1612–1620. [CrossRef] [PubMed]
25. Alonzo, S.H.; Stiver, K.A.; Marsh-Rollo, S.E. Ovarian fluid allows directional cryptic female choice despite external fertilization. *Nat. Commun.* **2016**, *16*, 12452. [CrossRef]
26. Yeates, S.E.; Diamond, S.E.; Einum, S.; Emerson, B.C.; Holt, W.V.; Gage, M.J. Cryptic choice of conspecific sperm controlled by the impact of ovarian fluid on sperm swimming. *Behav. Evol.* **2013**, *67*, 3523–3536. [CrossRef]
27. Beirão, J.; Purchase, C.F.; Wringe, B.F.; Fleming, I.A. Inter-population ovarian fluid variation differentially modulates sperm motility in Atlantic cod *Gadus morhua*. *J. Fish Biol.* **2015**, *87*, 54–68. [CrossRef]
28. Zadmajid, V.; Myers, J.N.; Sørensen, S.R.; Butts, I.A.E. Ovarian fluid and its impacts on spermatozoa performance in fish: A review. *Theriogenology* **2019**, *1*, 144–152. [CrossRef]
29. Splendiani, A.; Ruggeri, P.; Giovannotti, M.; Pesaresi, S.; Occhipinti, G.; Fioravanti, T.; Lorenzoni, M.; Nisi Cerioni, P.; Caputo Barucchi, V. Alien brown trout invasion of the Italian peninsula: The role of geological, climate and anthropogenic factors. *Biol. Invasions* **2016**, *18*, 2029–2044. [CrossRef]
30. Berrebi, P. Three brown trout *Salmo trutta lineages* in Corsica described through allozyme variation. *J. Fish Biol.* **2015**, *86*, 60–73. [CrossRef]
31. Sabatini, A.; Cannas, R.; Follesa, M.C.; Palmas, F.; Manunza, A.; Matta, G.; Pendugiu, A.A.; Serra,

P.; Cau, A. Genetic characteriza- tion and artificial reproduction attempt of endemic Sardinian trout *Salmo trutta* L., 1758 (Osteichthyes, Salmonidae): Experiences in captivity. *Ital. J. Zool.* **2011**, *78*, 20–26. [CrossRef]

32. Splendiani, A.; Fioravanti, T.; Giovannotti, M.; Olivieri, L.; Ruggeri, P.; Nisi Cerioni, P.; Vanni, S.; Enrichetti, F.; Caputo Barucchi, V. Museum samples could help to reconstruct the original distribution of *Salmo trutta* complex in Italy. *J. Fish Biol.* **2017**, *90*, 2443–2451. [CrossRef] [PubMed]
33. Di Iorio, M.; Esposito, S.; Rusco, G.; Roncarati, A.; Miranda, M.; Gibertoni, P.P.; Cerolini, S.; Iaffaldano, N. Semen cryopreservation for the Mediterranean brown trout of the Biferno River (Molise-Italy): Comparative study on the effects of basic extenders and cryoprotectants. *Sci. Rep.* **2019**, *9*, 9703. [CrossRef] [PubMed]
34. Rusco, G.; Di Iorio, M.; Gibertoni, P.P.; Esposito, S.; Penserini, M.; Roncarati, A.; Cerolini, S.; Iaffaldano, N. Optimization of Sperm Cryopreservation Protocol for Mediterranean Brown Trout: A Comparative Study of Non-Permeating Cryoprotectants and Thawing Rates In Vitro and In Vivo. *Animals* **2019**, *9*, 304. [CrossRef]
35. Rusco, G.; Di Iorio, M.; Iampietro, R.; Esposito, S.; Gibertoni, P.P.; Penserini, M.; Roncarati, A.; Iaffaldano, N. A Simple and Efficient Semen Cryopreservation Method to Increase the Genetic Variability of Endangered Mediterranean Brown Trout Inhabiting Molise Rivers. *Animals* **2020**, *10*, 403. [CrossRef] [PubMed]
36. Rusco, G.; Di Iorio, M.; Iampietro, R.; Roncarati, A.; Esposito, S.; Iaffaldano, N. Cryobank of Mediterranean Brown Trout Semen: Evaluation of the Use of Frozen Semen up to Six Hours Post-Collection. *Fishes* **2021**, *6*, 26. [CrossRef]
37. Ferguson, A.; Reed, T.E.; Cross, T.F.; McGinnity, P.; Prodöhl, P.A. Anadromy, potamodromy and residency in brown trout *Salmo trutta*: The role of genes and the environment. *J. Fish Biol.* **2019**, *95*, 692–718. [CrossRef]
38. Di Iorio, M.; Rusco, G.; Esposito, S.; D′Andrea, M.; Roncarati, A.; Iaffaldano, N. The role of semen cryobanks for protecting endangered native salmonids: Advantages and perspectives as outlined by the LIFE Nat.Sal.Mo. project on Mediterranean brown trout (Molise region-Italy). *Front. Mar. Sci.* **2023**, *9*, 1075498. [CrossRef]
39. Palombo, V.; De Zio, E.; Salvatore, G.; Esposito, S.; Iaffaldano, N.; D′Andrea, M. Genotyping of Two Mediterranean Trout Populations in Central-Southern Italy for Conservation Purposes Using a Rainbow-Trout-Derived SNP Array. *Animals* **2021**, *11*, 1803. [CrossRef]
40. Škraba Jurlina, D.; Maric´, A.; Mrdak, D.; Kanjuh, T.; Špelic´, I.; Nikolic´, V.; Piria, M.; Simonovic´, P. Alternative life-history in native trout (*Salmo* spp.) suppresses the invasive effect of alien trout strains introduced into streams in the Western part of the Balkans. *Front. Ecol. Evol.* **2020**, *8*, 188. [CrossRef]
41. Toews, D.P.; Brelsford, A. The biogeography of mitochondrial and nuclear discordance in animals. *Mol. Ecol.* **2012**, *21*, 3907–3930. [CrossRef]
42. Lorenzoni, M.; Carosi, A.; Giovannotti, M.; La Porta, G.; Splendiani, A.; Barucchi, V.C.

Ecology and conservation of the Mediterranean trout in the central Apennines (Italy). *J. Limnol.* **2019**, *78*. [CrossRef]

43. D′Agaro, E.; Gibertoni, P.; Marroni, F.; Messina, M.; Tibaldi, E.; Esposito, S. Genetic and Phenotypic Characteristics of the *Salmo trutta* Complex in Italy. *Appl. Sci.* **2022**, *12*, 3219. [CrossRef]
44. Polgar, G.; Iaia, M.; Righi, T.; Volta, P. The Italian Alpine and Subalpine trouts: Taxonomy, Evolution, and Conservation. *Biology* **2022**, *11*, 576. [CrossRef] [PubMed]
45. Purchase, C.F.; Rooke, A.C. Freezing ovarian fluid does not alter how it affects fish sperm swimming performance: Creating a cryptic female choice 'spice rack' for use in split-ejaculate experimentation. *J. Fish Biol.* **2020**, *96*, 693–699. [CrossRef] [PubMed]
46. Nynca, J.; Ciereszko, A. Measurement of concentration and viability of brook trout (*Salvelinus fontinalis*) spermatozoa using computer-aided fluorescent microscopy. *Aquacilture* **2009**, *292*, 256–258. [CrossRef]
47. Billard, R. Reproduction in rainbow trout: Sex differentiation, dynamics of gametogenesis, biology and preservation of gametes. *Aquaculture* **1992**, *100*, 263–298. [CrossRef]
48. Dadras, H.; Dzyuba, B.; Cosson, J.; Golpour, A.; Siddique, M.A.M.; Linhart, O. Effect of water temperature on the physiology of fish spermatozoon function: A brief review. *Aquac. Res.* **2016**, *48*, 729–740. [CrossRef]
49. Petersen, C.W.; Warner, R.R.; Cohen, S.; Hess, H.C.; Sewell, A.T. Variable pelagic fertilization success: Implications for mate choice and spatial patterns of mating. *Ecology* **1992**, *73*, 391–401. [CrossRef]
50. Petersen, C.W.; Warner, R.R. Sperm competition in fishes. In *Sperm Competition and Sexual Selection*; Birkhead, T.R., MØller, A.P., Eds.; Academic Press: San Jose, CA, USA, 1998; pp. 435–463.
51. Kime, D.E.; Van Look, K.J.W.; McAllister, B.G.; Huyskens, G.; Rurangwa, E.; Ollevier, F. Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. *Comp. Biochem. Physiol.* **2001**, *130*, 425–433. [CrossRef]
52. Yanagimachi, R.; Cherr, G.; Matsubara, T.; Andoh, T.; Harumi, T.; Vines, C.; Pillai, M.; Griffin, F.; Matsubara, H.; Weatherby, T. Sperm attractant in the micropyle region of fish and insect eggs. *Biol. Reprod.* **2013**, *88*, 1–11. [CrossRef]
53. Yanagimachi, R.; Harumi, T.; Matsubara, H.; Yan, W.; Yuan, S.; Hirohashi, N.; Iida, T.; Yamaha, E.; Arai, K.; Matsubara, T. Chemical and physical guidance of fish spermatozoa into the egg through the micropyle. *Biol. Reprod.* **2017**, *96*, 780–799. [CrossRef]
54. Lahnsteiner, F.; Berger, B.; Weismann, T.; Patzner, R.A. Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. *Aquaculture* **1998**, *163*, 163–181. [CrossRef]
55. Gage, M.J.G.; Macfarlane, C.P.; Yeates, S.; Ward, R.G.; Searle, J.B.; Parker, G.A. Spermatozoal traits and sperm competition in Atlantic salmon: Relative sperm velocity is the primary determinant of fertilization success. *Curr. Biol.* **2004**, *14*, 44–47. [CrossRef] [PubMed]
56. Linhart, O.; Rodina, M.; Gela, D.; Kocour, M.; Vandeputte, M. Spermatozoal competition in common

carp (*Cyprinus carpio*): What is the primary determinant of competition success? *Reprod. Colch.* **2005**, *130*, 705–711. [CrossRef] [PubMed]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Session 2

Preserving Avian Heritage: Establishing Italy's First Semen Cryobank for Native Chicken and Turkey Breeds

PREFACE

The increasing demand for poultry products reflects a steady increase in production. In Europe, despite a slight contraction in 2022, poultry production in 2023 remained at levels similar to 2018, ensuring self-sufficiency with a self-sufficiency rate exceeding 100%. Poland remains the top European producer, followed by Romania and Turkey. Spain has also seen an increase in production in recent years, while Italy, despite ranking fifth, has recorded more modest growth due to a saturated domestic market. Regarding egg production, France remains the leading producer with a share of 14% and production showing a growing trend, thanks to a 12% increase in laying hens raised in the last year. Germany, Spain, Italy, the Netherlands, and Poland follow suit (Isema 2023). At the same time, the industrialization of the poultry sector in many parts of the world has led to a greater prevalence of intensive farming systems. These systems rely exclusively on the use of high-yielding commercial hybrids to the exclusion of indigenous breeds (Delany, 2004). This trend has led to a significant reduction in the breeding of indigenous breeds.

According to data from the Domestic Animal Diversity Information System (DAD-IS), managed by the Food and Agriculture Organisation of the United Nations (FAO), a significant percentage of the local chicken breeds examined globally are classified as vulnerable or critically endangered, with some breeds already extinct (FAO 2020). In Italy, many of the 53 identified native chicken breeds are endangered or already extinct. However, there has been a considerable increase in interest in native breeds, both by the scientific community and the general public. Currently, 22 of these breeds are included in the National Biodiversity Conservation Project (Castillo et al. 2022). These breeds can offer an interesting alternative to commercial lines, providing high quality products of great interest for local and regional markets (De Marchi et al., 2005; Castellini et al., 2006). In fact, native chicken breeds offer significant advantages due to their adaptive characteristics to the geographical and environmental context, as well as their link with local traditions. These breeds require less human intervention and are suited to sustainable farming practices, with the ability to obtain food even in free-range systems. Furthermore, maintaining these breeds is crucial to preserving biodiversity and genetic diversity in the poultry sector, preparing it for future challenges (Bruijnis et al., 2015; Franzoni et al., 2021; Dal Bosco et al., 2021). The conservation of animal biodiversity is essential for maintaining ecological balance and ensuring vital ecosystem services. To achieve greater ecological stability, it is crucial to protect genetic resources and maintain high genetic variability that enables animals to adapt to climate change, respond to stress, and resist diseases. In Italy, the intensification of agriculture and the widespread use of commercial hybrids have led to a decline in native chicken breeds over the years. This has resulted in a significant loss of genetic variability, making animals less adaptable to rapid environmental changes and more susceptible to diseases (Zanon and Sabbioni,

2001; Castillo et al., 2021). The 2019 FAO report highlights a continuing decline in agricultural biodiversity, which poses risks to food production, livelihood sustainability, human health, and the environment. In response to this challenge, it becomes urgent to implement advanced conservation programs in poultry farming to protect the remaining biodiversity and provide the productive sector with genetic resources suitable for product diversification and the maintenance of evolving production systems, increasingly influenced by environmental and health issues.

The conservation of local breeds supports the development of rural economies in marginal agricultural areas and niche markets for high-quality poultry products.

In Italy, conservation projects aimed at safeguarding native poultry breeds have traditionally depended solely on the *in situ* technique, supported by regional and local public institutions. However, the TuBAvI project (www.pollitaliani.it) stands as a milestone, fostering coordinated conservation efforts at the national level. This initiative encompasses the establishment of the first Italian semen cryobank for autochthonous chicken and turkey breeds. The total semen doses stored within the Italian semen cryobank of autochthonous chicken and turkey breeds at the University of Molise and Milan amounted to 2298 (last update October 2023)

The main objective of the cryobank is to support the management of populations conserved *in vivo* and to potentially reconstruct breeds in the event of extinction or a significant reduction in population size. Currently, the creation of cryobanks for chicken semen has been addressed by some countries outside Europe (America, Japan) and in some European countries (France, Spain, Hungary, the Netherlands, and Switzerland). In contrast, cryobank for turkey semen is only found in North America and Hungary. However, these breeds are characterised by small, locally fragmented, and territory-bound populations, which are the main causes of the serious risk of genetic erosion. Consequently, as in other developed countries, the preservation of Italy's poultry biodiversity has become a matter of great concern.

In light of these considerations, the research activities reported in this session include two studies aimed at safeguarding genetic biodiversity in poultry and turkey breeds. The first study focuses on the importance of semen cryobanks of native breeds and their crucial role in linking *in situ* and *ex situ* techniques to improve the efficiency of conservation programmes. The second study aims to identify the molecular basis involved in the cryopreservation process of turkey spermatozoa, using metabolic profiling analysis by NMR spectroscopy on fresh and frozen samples, in order to develop new strategies to preserve the quality of frozen semen and genetic biodiversity.

References

- Bittante G. 2011. Italian animal genetic resources in the Domestic Animal Diversity Information System of FAO. Ital J Anim Sci. 10:151–158.
- Blesbois E, Grasseau I, Seigneurin F. 2005. Membrane fluidity and the ability of domestic bird spermatozoa to survive cryopreservation. Rev Reprod. 129(3):371–378
- 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.
- Blesbois, E.,2011. Freezing avian semen. *Avian biology research*, *4*(2), 52-58.
- Bruyn, J. D., Wong, J., Bagnol, B., Pengelly, B., & Alders, R. (2015). Family poultry and food and nutrition security.
- Burrows WH, Quinn JP. 1935. A method of obtaining spermatozoa from the domestic fowl. Poult Sci. 14:253–254.
- Castellini, C., & Dal Bosco, A. (2017). Animal welfare and poultry meat in alternative production systems (and ethics of poultry meat production). In *Poultry Quality Evaluation* (pp. 335-357). Woodhead Publishing.
- Castillo A, Gariglio M, Franzoni A, Soglia D, Sartore S, Buccioni A, Mannelli F, Cassandro M, Cendron F, Castellini C, et al. 2021. Overview of native chicken breeds in Italy: conservation status and rearing systems in use. Animals. 11(2):490.
- Cendron F, Perini F, Mastrangelo S, Tolone M, Criscione A, Bordonaro S, Iaffaldano N, Castellini C, Marzoni M, Buccioni A, Soglia D, et al. 2020. Genome-wide SNP analysis reveals the population structure and the conservation status of 23 Italian chicken breeds. Animals. 10(8):1441.
- Cerolini S, Vasconi M, Sayed AA, Iaffaldano N, Mangiagalli MG, Pastorelli G, Moretti VM, Zaniboni L, Mosca F. 2019. Free-range rearing density for male and female Milanino chickens: Carcass yield and qualitative meat traits. J Appl Poult Res. 28(4):1349–1358.
- Cerolini S, Zaniboni L, Mangiagalli MG, Gliozzi TM. 2007. Effect of glycine on cryopreservation of chicken spermatozoa. Av Poult Biol Rev. 18(2):65.
- Chalah T, Seigneurin F, Blesbois E, Brillard JP. 1999. In vitro comparison of fowl sperm viability in ejaculates frozen by three different techniques and relationship with subsequent fertility in vivo. Cryobiology. 39(2):185–191.
- Çiftci HB, Aygün A. 2018. Poultry semen cryopreservation technologies. World's Poult Sci J. 74(4):699–799.
- Dal Bosco, A., Mattioli, S., Cartoni Mancinelli, A., Cotozzolo, E., & Castellini, C. (2021). Extensive rearing systems in poultry production: The right chicken for the right farming system. A review of twenty years of scientific research in Perugia University, Italy. *Animals*, *11*(5), 1281.

- De Marchi, M.; Dalvit, C.; Targhetta, C.; Cassandro, M. Valutazione della diversità genetica nelle razze di pollo autoctone venete utilizzando marcatori AFLP. *Anima. Genetta.* 2006 , *37* , 101–105.
- Delany ME. 2004. Genetic variants for chick biology research: from breeds to mutants. Mech Dev. 121(9):1169–1177.
- Di Iorio M, Rusco G, Iampietro R, Colonna MA, Zaniboni L, Cerolini S, Iaffaldano N. 2020. Finding an effective freezing protocol for Turkey semen: benefits of ficoll as non-permeant cryoprotectant and 1:4 as dilution rate. Animals. 10(3):421.
- Directorate-General for Agriculture and Rural Development. Preparatory Action EU Plant and Animal Genetic Resources—Executive Summary; European Union: Luxembourg, 2020.
- Food and Agriculture Organization of the United Nations (FAO). *Domestic Animal Diversity Information System (DAD-IS)*; FAO: Rome, Italy, 2020; Available online: http://www.fao.org/dad-is/en (accessed on 20 December 2020).
- Food and Agriculture Organization of the United Nations (FAO). The State of the World's Animal Genetic Resources for Food and Agriculture; Chief, Electronic Publishing Policy and Support Branch Communication Division; FAO: Rome, Italy, 2007.
- Franzoni A, Gariglio M, Castillo A, Soglia D, Sartore S, Buccioni A, Mannelli F, Cassandro M, Cendron F, Castellini C, et al. 2021. Overview of native chicken breeds in Italy: small scale production and marketing. Animals. 11(3):629

STUDY 1

Italian Journal of Animal Science (2021)
(doi.org/10.1080/1828051X.2021.1993094)

ITALIAN JOURNAL OF ANIMAL SCIENCE
2021, VOL. 20, NO. 1, 2022–2033
https://doi.org/10.1080/1828051X.2021.1993094

Taylor & Francis
Taylor & Francis Group

REVIEW

OPEN ACCESS Check for updates

Italian semen cryobank of autochthonous chicken and turkey breeds: a tool for preserving genetic biodiversity

Nicolaia Iaffaldano[a], Michele Di Iorio[a], Giusy Rusco[a], Emanuele Antenucci[a], Luisa Zaniboni[b], Manuela Madeddu[b], Stefano Marelli[b], Achille Schiavone[c], Dominga Soglia[c], Arianna Buccioni[d], Martino Cassandro[e], Cesare Castellini[f], Margherita Marzoni[g] and Silvia Cerolini[b]

[a]Dipartimento Agricoltura, Ambiente e Alimenti, Università degli Studi del Molise, Campobasso, Italia; [b]Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, via dell'Università 6, Lodi, Italia; [c]Dipartimento di Scienze Veterinarie, Università degli Studi di Torino, Grugliasco, Italia; [d]Dipartimento di Scienze e Tecnologie Agrarie, Alimentari, Ambientali e Forestali, Università degli Studi di Firenze, Firenze, Italia; [e]Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente, Università degli Studi di Padova, Viale dell'Università 16, Legnaro, Italia; [f]Dipartimento di Scienze Agrarie, Alimentari e Ambientali, Università degli Studi di Perugia, Perugia, Italia; [g]Dipartimento di Scienze Veterinarie, Università-di Pisa, Pisa, Italia

ABSTRACT

The creation of genetic resource cryobanks provides a crucial link between *in situ* and *ex situ* techniques to improve the efficiency of conservation programs. Aim of the present review is to describe all the activities developed for the implementation of the first Italian Semen Cryobank of Autochthonous Chicken and Turkey Breeds. These activities can be classified into three main topics: (1) identification of species-specific semen freezing/thawing reference procedures; (2) drafting Standard Operative Procedures (SOP) for the implementation of the semen cryobank; (3) storage of semen doses from Italian chicken and turkey breeds to establish the cryobank. Several trials have been developed to identify a specie-specific semen cryopreservation protocol for chickens and turkeys. The major results are reviewed and a final reference protocol described. Taking into consideration the FAO guidelines for cryoconservation of animal genetic resources, SOP were drafted with the aim to provide technical guidance and logistical support on the choice of priority breeds, selection of birds for semen production, infrastructures and storage sites, birds and semen management, cryopreservation process and doses traceability. Lastly, the Italian Semen Cryobank was created. A total of 112 semen doses from 22 cockerels of three breeds, and 74 doses from 12 turkey males of three breeds were stored in the Cryobank. Breed specific semen quality parameters assessed before and after cryopreservation are reported. The described activities provide information and tools useful for the implementation of semen cryobanking in avian species and might be transferred also to other species after appropriate adaptations.

HIGHLIGHTS

- Implementation of the first Italian Semen Cryobank of Autochthonous Chicken and Turkey Breeds
- Drafting Standard Operative Procedures provides technical guidance and logistical support on the design and establishment of the cryobank
- Semen cryobank is a precious genetic reservoir and could be useful to safeguard genetic variability in small population *in vivo* conserved

ARTICLE HISTORY
Received 3 August 2021
Revised 28 September 2021
Accepted 10 October 2021

KEYWORDS
Gamete cryobanking; Italian poultry breeds; genetic resources; conservation program

CONTACT Dr. Michele Di Iorio michele.diiorio@unimol.it Dipartimento Agricoltura, Ambiente e Alimenti, Università degli Studi del Molise, Campobasso, Italia

© 2021 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

In the last few decades, in Italy as well as in the rest of the world, the poultry sector has suffered a significant loss in terms of animal genetic resources (AnGR) and a progressive erosion of many native genotypes due to the massive use of high-performing commer- cial hybrids (Delany 2004; Fulton 2006). The evolution of intensive farming has threatened the survival of local chicken and turkey breeds; in fact, the popula- tions of the native breeds are reared in very small numbers and may suffer from inbreeding and loss in genetic diversity (Zanon and Sabbioni 2001; Castillo et al. 2021). Safeguarding poultry biodiversity is a key objective in every developed country; almost no data are available on the wide avian genetic resources. reared in Italy and the urgent need of specific conservation programs for Italian poultry breeds is well recognised (Bittante 2011; Ozdemir et al. 2013). In this context, the project 'Conservation of biodiversity in Italian poultry breeds – TuBAvI' (www.pollitaliani.it) was developed from 2017 to 2020 with the financial support of Ministero delle Politiche Agricole Alimentari e Forestali (MIPAAF) and Fondo Europeo Agricolo per lo Sviluppo Rurale (FEASR). The TuBAvI project aimed to promote and support the conservation of the Italian poultry genetic resources providing new data and tools for the implementation of a wide comprehensive national conservation program. Conservation techniques can be divided into in situ and ex situ, and the latter can be further divided into in vivo and in vitro (FAO 2012; Leroy et al. 2019). The *in situ* technique involves the maintenance of the living animals within the livestock production system in which they were developed, whilst the *ex situ in vivo* involves the maintenance of the living animals outside their original area. The *ex situ in vitro* technique involves the cryopreservation of genetic material in haploid form (semen and oocytes), diploid (embryos, somatic cells) or DNA sequences (FAO 2012; Mara et al. 2013). Cryopreserved material is usually stored in specific facilities defined as gene banks or cryobanks, which are generally maintained by universities, research centres, government or private companies (FAO 2012; Leroy et al. 2019). The *in situ* conservation technique has clear priority, even because the animals continue to evolve in their original habitat; however, this approach is often too expensive as it requires dedicated infrastructures and proper management (Patterson and Silversides 2003; Prentice and Anzar 2011). Therefore, the development of the *ex situ in vitro* technique to be used as a support of the *in vivo* one is ever-increasing. The creation of genetic resource cryobanks would provide a crucial link between both techniques to improve the efficiency of conservation programs (Prentice and Anzar 2011).

In birds, the *ex situ in vitro* technique can rely only on semen cryopreservation, being the most suitable reproductive technology currently available for long storage of genetic resources (Long 2006; Blesbois 2011; Ehling et al. 2012). The cryopreservation of intact oocytes or embryos is not possible because of the characteristics of the megalecithal eggs (Long 2006; Blesbois et al. 2007; Blesbois

2011; Ehling et al. 2012). Technologies for the cryopreservation of gonadic tissues and primordial germ cells have been also developed during the last decade (Silversides et al. 2012; Liptoi et al. 2013; Sztan et al. 2017). However, they are still very expensive and highly invasive compared to semen cryopreservation (Nakamura 2016; Nandi et al. 2016).

In some European countries (France, Spain, The Netherlands, Hungary, Slovak Republic) and in United States of America, several conservation programs of AnGR have been developed and a national semen cryobank of local chicken breeds and/or specific genetic lines is currently being created (Szalay 2004; Woelders et al. 2006; Blesbois et al. 2007; Blackburn 2009; Santiago-Moreno et al. 2011). Meanwhile in Italy, conservation projects for the safeguard of native poultry breeds have been based only on the *in situ* technique and have been developed with the support of regional and local public institutions. For the first time, TuBAvI project allowed to develop common conservation activities cohordinated at national level, including the planning and implementation of the first Italian Semen Cryobank of Autochthonous Chicken and Turkey Breeds. The Cryobank is mainly aimed to support the management of populations in vivo con- served and the potential reconstruction of breeds in case of extinction or drastic reduction of their popula- tion size. The aim of the present report is to describe the activities developed and the rules adopted for the creation of the Cryobank: (1) identification of freezing/ thawing protocols for chicken and turkey semen; (2) drafting the Standard Operative Procedures (SOP) for the management of birds and semen from collection to storage; (3) storage in liquid nitrogen of semen doses in Italian chicken and turkey breeds.

Identification of the semen cryopreservation protocol in the *Gallus gallus* and *Meleagris gallopavo* species

The fundamental assumption for the realisation of a poultry semen Cryobank is the development of a suc- cessful freezing protocol. Even today, semen cryopreservation is still responsible for a severe loss in sperm integrity in the majority of domestic animals and the improvement of sperm cryosurvival and fertility after artificial insemination (AI) of frozen/thawed sperm is still the working focus in semen cryobanking for most mammalian and avian species. So far, the establishment of a sperm Cryobank for conservation of avian genetic resources has been usually associated with research trials to identify the most suitable freezing/thawing protocol able to preserve sperm integrity and fertilising ability (Woelders et al. 2006; Blesbois et al. 2007; Santiago-Moreno et al .2011; Thélie et al. 2019).

In order to identify a reference freezing/thawing protocol for chicken and turkey semen several trials have been developed to study the main factors involved in sperm cryosurvival: extenders, dilution rates, cryoprotectants (CPAs) and their concentration, freezing and thawing rates.

Although, chicken and turkey sperm share the same morphology i.e. both have peculiar physiological features such as a filiform shape, a long tail and a condensed nucleus, (Donoghue and Wishart 2000; Blesbois et al. 2005), however, the turkey sperm are generally recognised to be much more sensitive to freezing/thawing (Iaffaldano, et al. 2009; Di Iorio, Cerolini, et al. 2016; Iaffaldano, Di Iorio, Miranda, et al. 2016).

Specie-specific *in vitro* processing conditions have been tested and different protocols were finally identified according to the species.

Cryopreservation protocol for chicken semen - DiMethylacetamide (DM) is a permeant cryoprotectant successfully used for the cryopreservation of chicken semen packaged in pellets (Chalah et al. 1999; Tselutin et al. 1999; Zaniboni et al. 2014) and was also considered for straw packaging, according to FAO safety guidelines recommended in semen cryobanking (FAO 2012). Chicken semen packaged into straws was frozen in nitrogen vapours using very simple unexpensive floating racks, and the most suitable temperature gradient during freezing was studied. Rapid freezing obtained by exposure of straws to vapours 3 cm above the liquid nitrogen bath allowed to reach -40°C within 1 min and was confirmed to be the most suitable gradient for freezing chicken semen packaged into straws (Madeddu et al. 2016). The cryoprotective action of DM was further improved in association with the non-permeant cryoprotectant trehalose, not with sucrose, playing a synergic action with DM able to improve sperm kinetic parameters and the recovery of progressive motile sperm from 15% to 24% after freez- ing/thawing (Mosca et al. 2016a). If combined with trehalose, DM concentration was reduced from 6% to 3% with no negative effect on sperm viability (32%), motility (24%) and progressive motility (1.4%) after freezing/thawing; however, trehalose alone did not provide an adequate cryoprotection and could not completely replace DM (Mosca et al. 2016b).

The research activities developed during the TuBAvI project have been focussed on the action of the CPAs on both sperm integrity, assessed in vitro, and embryo viability, assessed in vivo. DM was compared with N- Methylacetamide (NM), a new permeant cryoprotectant for chicken semen used with excellent fertility (77–84%) and hatchability (90%) results in the Yakido rare breed (Sasaki et al. 2010). Different DM and NM concentrations, from 2% to 9%, were tested and compared in the same trials. Both cryoprotectants showed a similar cryoprotective action on sperm integrity and function according a clear concentration dependent positive effect within the range 2–6% final concentra- tion: sperm membrane integrity, motility and progres- sive motility recorded in frozen/thawed semen in presence of 6% CPA were 39%, 53% and 11% respectively (Zaniboni et al. 2021). A further increase to 9% CPA concentration was associated with a further improvement in sperm membrane integrity with DM, not NM (Mosca et al. 2019, 2020). In contrast, semen cryopreserved with DM and NM provided different results after artificial insemination according to

the CPA. Despite similar fertility rates, the occurrence of embryo development was confirmed with NM-treated semen, not DMA, in following trials (Mosca et al. 2019; Zaniboni et al. 2021). In presence of NM, a negative relation between CPA concentration and fertility was found, being the highest fertility rate (9%) recorded with 2% NM, whereas the proportion of viable embryos/fertile eggs (recorded on day 7 of incubation) was not affected by CPA concentration and 49% over- all mean was recorded (Zaniboni et al. 2021). In contrast in presence of DM, fertility and embryo viability were positively affected by the CPA concentration and the highest proportion of fertile eggs (9%) and viable embryos (47% on fertile eggs) required the 6% DM (Zaniboni et al. 2021). The results suggest a higher protective action, or a higher permeability to sperm membrane, of NM compared to DM, being able to provide fertilisation and embryo development at lower concentration. The cryoprotective action of NM on sperm integrity and motility was also affected by the thawing temperature. Cold thawing, corresponding to 5°C for 100 s, was less harmful compared with warm thawing, corresponding to 38°C for 10 s, and the proportions of sperm with undamaged plasma membrane, motile and progressive motile sperm in frozen/thawed samples were 51%, 52% and 11% respectively (Mosca et al. 2020).

The concentration of the insemination dose of cryopreserved chicken semen was also studied to identify the lowest dose able to provide fertile eggs and viable embryos in order to optimise the use of frozen/ thawed semen in artificial insemination protocols. A single insemination of 250, 500 and 750 × 10^6 frozen/ thawed sperm provided a similar proportion of viable embryos (average 16% on egg set) from day 2 to day 4 after insemination, irrespective of the concentration dose. However, longer fertile period was recorded with 500 and 750 × 10^6 insemination dose of frozen/ thawed sperm (Cerolini et al. 2019). The insemination of 250 × 10^6 sperm/dose is suggested in artificial insemination protocols with cryopreserved semen in order to optimise semen management and obtain maximum fertility with the lowest dose.

The following cryopreservation protocol was finally identified for *in vitro* processing of chicken semen:

- Dilution to 1.5 × 10^9 sperm/mL with Modified pre- Freezing Lake (MFL) diluent (Mosca et al. 2016a);
- Cooling at 4°C for 20 min;
- Dilution at 1.0 × 10^9 sperm/mL with MFL diluent added with NM 2% final concentration;
- Equilibrium at 4 ◦C for 1 min;
- Packaging into straws (0.25 mL): 250 × 10 sperm/straw;
- Freezing by exposure of straws 3 cm above liquid nitrogen bath for 10 min;

- Transfer and storage of straws into liquid nitrogen cryotank;
- Thawing in a thermostatically controlled bath at 5°C for 100 s.

Cryopreservation protocol for turkey semen - A promising freezing protocol for turkey semen was identified by testing different critical steps of in vitro processing, such as the choice of the permeant CPA and its concentration, and the freezing and thawing rates. The best protocol identified involved the use of 10% DMSO, semen freezing by exposure of straws at 10 cm above a liquid nitrogen bath and thawing at 50°C for 10 s (Iaffaldano, Di Iorio, Miranda, et al. 2016). However, despite the protocol provided encouraging results, corresponding to 37% motility, 42% viability and 25% osmotic tolerance in frozen/thawed semen samples, further efforts were needed in order to further improve and standardise the cryopreservation protocol to be implemented in the Cryobank.

The research activities developed within the TuBAvI project were aimed to study the action of two dilution rates (1:2 and 1:4) and the effects of three non-perme- ant-cryoprotectants (sucrose, trehalose and Ficoll 70) at four different concentrations in combination with DMSO on *in vitro* post-thaw semen quality (Di Iorio, Rusco, Iampietro, Colonna, et al. 2020). The rationale of this study was to test the beneficial effect of the combined use between permeant and non-permeant cryoprotectants, according to literature reports (Blanco et al. 2011; Iaffaldano, Di Iorio, Cerolini, et al. 2016; Mosca et al. 2016a; Miranda et al. 2018). Among all combinations tested the best results in terms of sperm cryosurvival (32.6% total motility; 3.5% progressive motility; 42.5% membrane integrity; 24.4% osmotic resistance and 97.9% DNA integrity) were recorded with the use of 1 mM Ficoll and the 1:4 dilution rate. In order to validate the most effective freezing protocol, identified by in vitro assessments of semen quality, frozen/thawed semen was also used for artificial insemination to assess in vivo fertility and hatchability (Di Iorio, et al. 2020). Different concentrations of the insemination dose were tested in female turkey breeders showing a clear effect on fertility and hatching rates.

Hens were inseminated both using fresh and frozen semen twice every 7 days. The best results of fertility and hatchability (inseminating dose of 400×10^6 sperm/hen) were 87% and 71% respectively using frozen semen compared to 90.8 and 75.6% respectively using fresh semen (Di Iorio et al. 2020).

The following cryopreservation protocol was finally identified for in vitro processing of turkey semen:

- Pre-dilution to 6×10^9 sperm/mL with Lake diluent (Di Iorio et al., 2020);
- Cooling to 4°C for 25 min;
- Dilution 1:1 (*v:v*) at 3×10^9 sperm/mL with freezing Lake extender + 20% DMSO + 1mM Ficoll;
- Packaging in straws (0.25 mL): 750×10^6 sperm/straw;
- Equilibration at 4°C for 20 min;

- Freezing by exposure of straws 10 cm above liquid nitrogen bath for 10 min;
- Transfer and storage of straws into liquid nitrogen cryotank;
- Thawing in a thermostatically controlled bath at 50°C for 10 s.

Standard Operative Procedures (SOP) for theimplementation of the Semen Cryobank

In order to realise the first Italian Semen Cryobank of autochthonous chicken and turkey breeds, Standard Operative Procedures have been discussed taking into consideration the FAO guidelines on the management of small populations and guidelines on animal genetic resources cryobanking (FAO 2009, 2012). The main aspects dealt with were: choice of priority breeds, selection and management of semen donor males, semen management and cryopreservation processing, infrastructures and storage sites.

The breed priority for ex situ in vitro conservation is based on the knowledge of the status of the breed or breeding population's potentiality. However, very few data were available about genetic, breeding and farming characteristics of Italian poultry breeds. In order to fill this gap, TuBAvI project developed several activities for breed characterisation, including the census of Italian poultry breeds still reared in farming systems (Castillo et al. 2021; Franzoni et al. 2021) and the study of genetic diversity in the in vivo populations (Cendron et al. 2020; Soglia et al. 2021).

The TuBAvI census confirmed the presence of 18 chicken breeds and 7 turkey breeds in the different Italian regions of the Country (Castillo et al. 2021), cor- responding to the large majority of the breeds included in the Poultry Breed Herd Book (www.anci- aia.it). However, the total population size was less than 500 birds in the majority of the breeds and was above 1000 birds in only 4 chicken breeds (Bionda piemontese, Livorno, Padovana, Polverara) (Castillo et al. 2021). In agreement with the 7 FAO risk categories, established according the overall population size, the number of breeding females and the trend in population size (FAO 2003), most of the Italian chicken and turkey breeds can be classified in the critical category and a small number in the endangered category. Therefore, all Italian chicken and turkey breeds are considered as priorities for ex situ in vitro germ- plasm conservation.

The study on genetic diversity between and within breeds revealed a large variation among breeds in the level of genomic inbreeding, investigated using Run of Homozygosity (ROH) data, and very few breeds showed a low level with $F_{ROH} < 0.1$ (Cendron et al. 2020). The average population molecular kinship was 53% and the mean inbreeding rate 56% with self- coancestry of 78% as a consequence of the reduced population size and genetic drift; the Livorno, Robusta Maculata, Robusta Lionata, Pepoi and Ermellinata di Rovigo breeds were recognised the most endangered populations (Soglia et

al. 2021). The need to implement the *ex situ in vitro* technique to support in vivo breed conservation was then confirmed.

Semen donors will be selected according to different characteristics: (a) morphology; (b) genetic diversity; (c) health status; (d) semen quality. The morphological characterisation will be performed recording qualitative and quantitative traits, according to FAO guidelines for phenotypic characterisation (2012). Phenotypic traits has to refer to the breed standard, as reported in the Poultry Breed Herd Book, and birds carrying undesired traits will be discarded. In addition, male donors should have the lowest possible degree of kinship, assessed according pedigree records, if available. In breed populations where bird genotyping with microsatellite markers is planned to apply mating scheme, selection of birds with high individual genetic variability will be also considered. The health status of birds is of relevant importance in order to avoid the vertical transmission of diseases to the future generations. Semen donors have to be vaccinated against the most severe infective poultry diseases (Marek and Newcastle diseases) and tested to be free of the following diseases: mycoplasmosis (*M. gallisepticum*, *M. synoviae*, *M. meleagridis*), salmonellosis, pullorum disease/fowl typhoid (*S. pullorum and gallinarum*) and avian influenza.

The plan to implement the Cryobank is mainly aimed to support the management of populations *in vivo* conserved. According to FAO guidelines n. 12 (2012), from 3 to 20 birds within breed will be selected and at least 20 semen doses per bird will be stored; the number of semen doses per bird could be modified according to the number of semen donors available in order to collect almost 400 semen doses per breed. Semen doses will be collected from the majority of the Italian chicken and turkey breeds during the lifetime of TuBAvI-2 project.

Semen management is described in section ‘Creation of the semen Cryobank’; the protocol for cryopreservation of chicken and turkey semen and the concentration dose of frozen/thawed semen for artificial insemination are described in section ‘Introduction’.

According to FAO guidelines (2012), the cryobank could be constructed in existing infrastructures, to carry out all of the activities, such as: (1) collection of the semen, (2) semen processing and freezing and (3) sperm dose storage. It is necessary to have different buildings suitable for carrying out these activities although, having all infrastructures in the same place is not strictly necessary.

In most cases the facilities will belong to commercial organisations (AI centre, university, research centres, industry), but in specific cases they could be private facilities that belong to individual breeders or non-profit organisations (ERFP, 2003).

The semen collection must be performed in an ani- mal holding facility while the semen evaluation and freezing occurs in a specific laboratory.

The infrastructure and expertise required for the creation of the Cryobank are provided by the University of Milan and the University of Molise, being both TuBAvI partners, and the same universities will be also the storage sites of semen doses.

Creation of the semen Cryobank

2. Materials and methods

Data were processed according to descriptive statistics using the MS Excel software. Mean values and their variability (SD) are presented.

Birds management and semen collection

Birds from different breeds within the *Gallus gallus* and *Meleagris gallopavo* species have been used to start-up the Italian Semen Cryobank. The chicken breeds were: Bianca di Saluzzo (BS, n=18), Bionda Piemontese (BP, n=25), Mericanel della Brianza (MB, n=9); the turkey breeds were: Romagnolo (RO, n=6), Ermellinato di Rovigo (ER, n=2), Bronzato Comune (BC, n=5). Cockerels were housed indoor in individual cages (40 × 50 cm) in controlled environment at the Animal Production Centre, University of Milan (Lodi). Turkeys were raised in outdoor pens in a private breeding farm (Masseria Paglicci, Rignano Garganico, Foggia). All birds were fed *ad libitum* a standard commercial breeder diets (16% CP, 2800 kcal ME/kg) and drinking water. Birds were fasted before semen collection.

Semen was routinely collected twice weekly with the consolidated technique of the abdominal massage (Burrows and Quinn 1935), after a training period ranging from 2 to 4 weeks. A first macroscopic assessment of semen quality was performed soon after collection and only ejaculates with homogeneous white opalescent appearance and high viscosity were kept for further analyses and *in vitro* processing. The handling of animals and semen collection was conducted in accordance with the Code of Ethics of the EU Directive 2010/63/EU.

Semen quality assessment and cryopreservation

The quality of the ejaculates was assessed soon after collection by the measurement of volume, concentration, sperm membrane integrity (SMI), total motility (TM) and progressive motility (PM). Volume was measured with calibrated micropipette. Sperm concentration was measured after 1:200 dilution in 0.9% NaCl using a calibrated photometer (IMV, L'Aigle, France) at a wavelength of 535 nm (Brillard and McDaniel 1985). In the turkey, sperm membrane integrity (SMI) was assessed by means of the Muse® Cell Analyzer (Luminex corporation, 12212 Technology Blvd Suite 130, Austin, TX 78727, United States) following the manufacturer's protocol. Semen samples were extended in PBS to reach a concentration ranging from 1×10^5 to 1×10^7 spermatozoa/mL. Then, 20 µL of this

suspension was mixed with 780 µL (dilution factor 1:40) of Muse Count & Viability Kit® in an Eppendorf tube (Luminex corporation) and incubated for 5 min at room temperature in the dark. Subsequently, the sperm suspension was analyzed by flow cytometry. Then, the Software Module performed calculations and displayed data in two dot plots: 1. nucleated cells; a membrane-permeant DNA staining dye that stained all cells that had a nucleus. This plots function is to identify cells with a nucleus from debris and non-nucleated cells. 2. Viability; a DNA-binding dye stains cells that had lost their membrane integrity and allowed the dye to stain the nucleus of dead and dying cells. This parameter discriminates viable (live cells that do not stain) from non-viable (dead or dying cells that stain). In the chicken, sperm membrane integrity was measured using the SYBR14/PI (propidium iodide) dual staining procedure (LIVE/DEAD SpermViability Kit, Molecular Probes, Invitrogen), as described by Rosato and Iaffaldano (2011) with minor modifications (Mosca et al. 2020). Total sperm motility (TM) and progressive motility (PM) were assessed by means of a computer-aided sperm analysis system coupled to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast) using the Sperm Class Analyzer (SCA) software (version 4.0, Microptic S.L., Barcelona, Spain). Semen samples were diluted with 0.9% NaCl to 100×10^6/mL concentration, incubated for 5 min at 38°C (turkey semen) or 20 min at room temperature (chicken semen) and then a semen aliquot was analysed under the microscope at 100× total magnification to record the proportion of motile (%) and progressive motile sperm (%).

After the quality assessment, ejaculates were processed for cryopreservation according to the protocol described in section 1. Sperm membrane integrity, motility and progressive motility were measured in frozen/thawed semen also. After at least 7 days storage, semen was thawed according the procedure described in section 1 and quality parameters assessed as previously described.

An appropriate code system was adopted to guarantee the traceability of semen doses. The code system has originated as follows: each straw was given a code (ID) associated with male information (i.e. breed, age, origin, etc.); straws from each male were collected in the same cryo-goblet marked with the semen donor ID and the date of freezing. An excel database was created to organize all the data related to the stored semen doses.

3. Results

Training to semen collection was rather difficult in cockerels and ejaculation was absent or very poor in many birds. The males selected for semen production were only 7 (28% total males) in the BP breed, 6 (33% total males) in the BS breed and 6 (67% total males) in the MB breed. In contrast, toms were easily trained to semen collection and almost all birds have been used for semen production.

Semen quality parameters recorded in fresh semen of all breeds are reported in Table 1. In chicken breeds, mean ejaculate volumes and concentrations were within the standard range peculiar of the species (Marzoni 2008), with the exception of a low ejaculate volume in the MB breed, probably related to the small size of the birds, having a mean body weight of 900 g. TM, PM and SMI showed higher mean values in the ejaculates of BP and MB breeds compared to the ejaculates of BS breed. In turkey breeds, ejaculate volume was within the standard range values peculiar of the species, whereas lower sperm concentration was recorded (Marzoni 2008). Higher values of semen volume were found in the RO breed, whilst a higher sperm concentration was recorded in the ER breed. TM values were very similar in ejaculates of all turkeys. Similar values of PM and SMI were found in ejaculates of RO and BC breeds that resulted as lower in ER breed.

As expected, the semen cryopreservation process caused a significant deterioration in sperm quality, in agreement with our previous studies both in chickens (Madeddu et al. 2016; Mosca et al. 2016a, 2019) and turkeys (Iaffaldano et al. 2016b, Di Iorio et al. 2020 a,b). In chickens, quality parameters recorded in frozen/thawed semen of BS and BP breeds were similar, even if the quality of fresh semen was different between breeds, whereas higher values in semen quality parameters were recorded in the MB breed (Table 2). Severe loss in SMI, TM and PM was observed in all chicken breeds and the most severe damage, corresponding to the loss of almost 90%, was recorded in the proportion of PM. In turkeys, a general loss of 70 % of TM and over 90 % of PM was observed (Table 2).

The census of the semen Cryobank, started within the TuBAvI project is shown in Table 3.

Table 1. Quality of fresh semen (means ± SD) in chicken and turkey Italian breeds: Romagnolo (RO), Bronzato Comune (BC), Ermellinato di Rovigo (ER), Bianca di Saluzzo (BS), Bionda Piemontese (BP), Mericanel della Brianza (MB).

Breed	**Semen parameters***				
	Vol	**Conc**	**TM**	**PM**	**SMI**
			Gallus gallus		
BS	330 ± 110	2.89 ± 0.62	62.75 ± 17.18	13.45 ± 0.07	65.77 ± 1.05
BP	250 ± 170	3.77 ± 0.76	86.07 ± 12.17	19.05 ± 5.73	84.21 ± 11.56
MB	106 ± 13	2.54 ± 0.49	87.60 ± 7.19	26.60 ± 10.02	96.01 ± 2.62
			Meleagris gallopavo		
RO	190 ± 88	5.81 ± 1.31	76.64 ± 6.24	21.47 ± 7.04	95.90 ± 1.03
BC	150 ± 50	5.51 ± 1.08	76.20 ± 8.10	22.31 ± 7.23	93.40 ± 1.42
ER	112 ± 18	6.27 ± 0.74	73.81 ± 0.52	15.35 ± 1.34	88.73 ± 3.31

* Vol: volume (μL); Conc: concentration ($\times 10^9$/mL), TM: total motile sperm (%); PM: progressive motile sperm (%); SMI: sperm membrane integrity (%).

Table 2. Quality of frozen/thawed semen (means ± SD) in chicken and turkey Italian breeds: Romagnolo (RO), Bronzato Comune (BC), Ermellinato di Rovigo (ER), Bianca di Saluzzo (BS), Bionda Piemontese (BP), Mericanel della Brianza (MB).

Breed	Semen parameters*		
	TM	PM	SMI
	Gallus gallus		
BS	17.45 ± 7.37	0.83 ± 1.83	15.55 ± 5.61
BP	16.98 ± 5.21	1.50 ± 1.83	17.24 ± 5.61
MB	30.70 ± 13.81	4.97 ± 2.38	33.55 ± 13.52
	Meleagris gallopavo		
RO	22.38 ± 0.10	2.57 ± 1.38	51.50 ± 7.40
BC	23.72 ± 3.10	1.88 ± 0.68	44.33 ± 1.42
ER	14.64 ± 1.28	0.89 ± 0.31	37.38 ± 4.46

*TM: total motile sperm (%); PM: progressive motile sperm (%); SMI: sperm membrane integrity (%).

Table 3. Semen doses stored within the Italian Semen Cryobank of autochthonous chicken and turkey breeds at the University of Molise and Milan

Species	Breeds	N° of donors	N° of doses
Gallus gallus	Bianca di Saluzzo	6	32
	Bionda Piemontese	7	67
	Mericanel della Brianza	9	13
Meleagris gallopavo	Romagnolo	5	50
	Bronzato	2	13
	Ermellinato di Rovigo	5	11

4. Discussion

In recent years, the interest in the conservation of animal biodiversity has intensified due to the risk of extinction of many native breeds, including in particular avian species. The awareness of the importance in conserving AnGR is demonstrated by the increasing numbers of conservation programmes developed in recent decades in Europe and worldwide (FAO 2015).

Gene banks represent the primary tool for conservation of AnGR according the *in vitro* technique. It has been estimated at global level that at least 128 countries have or are starting gene banks for preserving livestock genetic resources (FAO 2015). Paiva et al. (2016) reported that the global collection of AnGR probably exceeds 67,000 animals and about 4 million types of germplasm/tissue, and among these a consistent proportion is represented by semen cryobanks.

Semen cryobanking is aimed to several relevant purposes: a) reconstruction of breeds in case of extinction because of catastrophic events (i.e. disease, climatic adversities), b) as a back-up to quickly modify the selection process of populations and/or in case of genetic problems in the *in vivo* conserved populations, c) to increase effective population size and reduce genetic drift, d) research investigations (Gandini and Oldenbroek 2007).

Moreover, semen cryobanks can also supply high quality and safe semen as reservoir of genetic traits of interest (performance, resistance to diseases and/or parasites, behavioural traits related to adaptability and welfare) to be introduced in commercial productions or reintroduced after their loss during selective procedures in hybrids' creation.

The semen cryobank realised within our project is yet in the embryonic phase, however we aim to obtain an open cryobank that could serve as a service for breeders by providing them with semen doses from males with a high genetic value and at the same time contributing to the control of inbreeding on farms.

In accordance to FAO guidelines (FAO 2012), when the realization of a semen Cryobank is approached the following three principles should be considered: 1) to conserve small amounts of germplasm from many donor animals rather than large amounts from few donors; 2) to choose donors that are as genetically and phenotypically different in order to represent the genetic diversity of the population as much as possible; 3) to store the breeds as pure lines rather than gene pools to allow the use of the unique combinations of traits and the flexibility of stock combination. The Italian Semen Cryobank of autochthonous chicken and turkey breeds is going to be a precious genetic tank to support the management of *in vivo* conserved populations. Integrated live and cryopreserved schemes are aimed to re-establish the populations in case of physical disaster or genetic problems and are highly recommended if the effective population size does not exceed 50 (Meuwissen 2017). Conservation programs of Italian poultry populations, characterized by high risk status, will take advantage of the potential application of integrated live and cryopreserved schemes.

Semen cryobanks result as more convenient in the long-term than *in vivo* conservation programmes. In the late 1990s, it was announced that gene banking was more expensive than maintaining *in vivo* populations (FAO 1998). However, it has been proven over a 20-years' time horizon that gene banks reduce the conservation costs of an avian population by over 90% compared to *in vivo* conservation (Silversides et al. 2012).

The main challenge for the creation of a sperm Cryobank was the identification of an effective cryopreservation protocol that is able to guarantee both at least 25-30% of live/motile spermatozoa after the freezing/thawing process and sperm fertilization followed by embryo development. The identification of an effective freezing protocol for avian semen is particularly difficult because avian

sperm are more sensitive to freezing damages than mammalian sperm, as a consequence of their unique morphological features. In fact, avian sperm are characterized by a filiform shape due to the cylindrical head not much wider than the tail, a more condensed nucleus, almost no cytoplasmic volume and a very long tail approximately eight times the head length (Donoghue and Wishart 2000; Long 2006). In birds, semen cryopreservation is not a standardized procedure and its success is still greatly variable and dependent on the species, the genetic types/breeds within the species and the *in vitro* processing.

Cryopreservation *in vitro* processing involves several steps and each one affects sperm structure and functions (Garner et al. 1999; Bailey et al. 2003; Iaffaldano et al. 2016a,b). Deleterious effects are the result of osmotic stress and temperature changes produced during cooling, freezing and rewarming, being ice crystal formation the main biophysical mechanism responsible for cell death (Swain and Smith 2010).

The specie-specific freezing/thawing protocols identified for the implementation of the Italian Semen Cryobank have been developed according to the results of several investigations aimed to study the effect on sperm quality and fertilizing ability of many steps involved in *in vitro* processing: mainly the extender composition (Cerolini et al. 2007; Di Iorio et al. 2020b), the type and concentration of the permeant CPA (Iaffaldano et al. 2016b; Mosca et al. 2019; Zaniboni et al. 2021) and the addition of non-permeant CPA (Mosca et al. 2016a,b; Di Iorio et al. 2020a), the freezing and thawing rates (Iaffaldano et al. 2016b; Madeddu et al. 2016; Mosca et al. 2020). In the chicken, despite a high proportion of viable and motile sperm recovered after thawing (Mosca et al. 2020), a low proportion of fertile eggs and viable embryos were recorded after AI of thawed semen (Mosca et al. 2019; Zaniboni et al. 2021). Fertility of chicken sperm cryopreserved in presence of NM is highly variable and the range 0-100% is reported according to the breed/line (Sasaky et al. 2010; Lee et al. 2012; Kim et al. 2014; Shannungan et al. 2018; Pranay Kumar et al. 2018). The storage of germplasm from many different chicken breeds within the Italian Cryobank is of great scientific interest allowing a wide investigation on the relation between breeds and sperm sensitivity to cryopreservation that might provide new strategies to improve integrity and fertilizing ability of frozen/thawed semen. In the turkey, high proportions of viable and motile sperm were recorded after thawing (Iaffaldano et al. 2016b; Di Iorio et al. 2020a) and also high proportions of fertile eggs and hatched birds after AI of thawed semen (Di Iorio et al. 2020b). For the first time, a successful cryopreservation protocol is proposed for semen cyobanking in the *Meleagris gallopavo* species and the positive results are expected to be confirmed in the Italian breeds. Very few data on fertility rate of frozen/thawed turkey semen packaged into straws were previously reported and reviewed (Çiftci and Aygun 2018).

The draw up of SOP provided technical guidance and logistical support for the establishment of the Cryobank. The guidelines represents an handbook for the implementation of the semen Cryobank of Italian chicken and turkey breeds and the final official document will be published in the TuBAvI project website (www.pollitaliani.it). The SOP have been prepared following recommendations of the FAO Guidelines 'Cryoconservation of AnGR' (2012) that have been supplemented according the activities and results developed within TuBAvI project. The SOP identified for the Italian Semen Cryobank might provide interesting hints and support the development of semen cryobanking for poultry breeds by other universities, research centres or private companies.

A critical point emerged from the beginning of semen cryobanking was the limited number of birds available within breed populations. The results of the recent TuBAvI census showed that most of the Italian chicken breeds (n = 10) and all Italian turkey breeds (n = 7) have a total population of less than 500 birds (Castillo et al. 2021), and the size of the populations could be also more critical when referred only to breeding birds. The very small population size of Italian poultry breeds inevitably reduces the amount of genetic resources available for the selection of male breeder semen donors and, as a consequence, for semen storage in the Cryobank. Furthermore, the proportion of birds successfully trained to the handling required for semen collection was rather low in BS (33%) and BP (28%) chicken breeds, less critical in MB chicken breed and unexpectedly successful in turkey breeds. FAO guidelines (FAO 2012) suggests to collect semen from a minimum of 25 donors per breed and the required number of straws for the reconstitution process (five generations backcross) of a chicken breed was calculated to correspond to 513 (FAO 2012). However, in critical conditions due to very small populations and/or limited budget, the recommendation is to collect as much germplasm as possible and collecting germplasm from fewer than 25 animals from each available breed may be preferred over collecting germplasm from 25 animals from a lower number of breeds.

According to FAO recommendations and the general high risk of biodiversity erosion in Italian poultry breed populations, the Italian Semen Cryobank of autochthonous chicken and turkey breeds will be developed storing germplasm of as much as possible breeds even if the number of birds within breed will be limited. The storage of semen doses from 15 chicken breeds and 4 turkey breeds, representing the large majority of the breeds included in the Italian Herd Book, has been planned in the next three years and birds will be preferably selected within the populations maintained within Public Poultry Conservation Centers.

5. Conclusions

The establishment of the first Italian Semen Cryobank of autochthonous chicken and turkey breeds represents a precious tool for the safeguard and conservation of biodiversity in the Italian poultry

breed populations still present in farming systems. The Italian Semen Cryobank will allow the application of integrated *in vivo* and cryopreserved schemes to overcome genetic problems and prevent the extinction of breeds. The conservation of local breeds support the development of rural economies in marginal agricultural areas and niche markets for high-quality poultry products.

Efforts have been already planned to fully implement the Cryobank and store genetic resources from several different breeds. The plan includes the storage of semen doses from 15 chicken breeds and 4 turkey breeds representing the large majority of the breeds included in the Italian Poultry Herd Book. The activities reported in the present paper for the establishment of the Italian avian Semen Cryobank represent general guidelines that might be transferred to similar initiatives in birds and/or in different domestic animals.

Disclosure statement

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

Additional information

Funding

This study was funded by Ministry of Agricultural, Food, Forestry and Tourism Policies (PSRN 2014-2020 Sottomisura 10.2 Biodiversità/Comparto avicoli - TuBAvI project n. 54250333926, with FEASR support).

Data availability statement (DAS)

The datasets used are available from the corresponding author on reasonable request.

References

1. Bailey JL, Morrier A, Cormier N. 2003. Semen cryopreservation: success and persistent problems in farm species. Can J Anim Sci. 83: 393−401.
2. Bittante G. 2011. Italian animal genetic resources in the Domestic Animal Diversity Information System of FAO. Ital J Anim Sci. 10:151-158.
3. Blackburn HD. 2009. Genebank development for the conservation of livestock genetic resources in the United States of America. Livest Prod Sci. 120:196–203.
4. Blanco JM, Long JA, Gee G, Wildt DE, Donoghue AM. 2011. Comparative cryopreservation of avian spermatozoa: Benefits of non-permeating osmoprotectants and ATP on turkey and crane sperm cryosurvival. Anim Reprod Sci. 123:242–248.
5. Blesbois E, Grasseau I, Segineurin F. 2005. Membrane fluidity and the ability of domestic bird spermatozoa to survive cryopreservation. Rev Reprod. 129(3):371–378.
6. Blesbois E, Seigneurin F, Grasseau I, Limouzin C, Besnard J, Gourichon D, Coquerelle G, Rault P, Tixier-Boichard M. 2007. Semen cryopreservation for ex situ management of genetic diversity in chicken: Creation of the French avian cryobank. Poult Sci. 86:555–564.

7. Blesbois E. 2011. Freezing avian semen. Avian Biol Res. 4:52–58.
8. Brillard JP, McDaniel GR. 1985. The reliability and efficiency of various methods for estimating spermatozoa concentration. Br Poult Sci. 64:155-158.
9. Burrows WH, Quinn JP. 1935. A method of obtaining spermatozoa from the domestic fowl. Poult Sci. 14:253–254.
10. Castillo A, Gariglio M, Franzoni A, Soglia D, Sartore S, Buccioni A, Mannelli F, Cassandro M, Cendron F, Castellini C, Mancinelli Cartoni A, Iaffaldano N, Di Iorio M, Marzoni M, Salvucci S, Cerolini S, Zaniboni L, Schiavone A. 2021. Overview of native chicken breeds in Italy: conservation status and rearing systems in use. Animals. 11:490.
11. Cendron F, Perini F, Mastrangelo S, Tolone M, Criscione A, Bordonaro S, Iaffaldano N, Castellini C, Marzoni M. Buccioni A, Soglia D, Schiavone A, Cerolini S, Lasagna E, Cassandro M. 2020. Genome-wide SNP analysis reveals the population structure and the conservation status of 23 Italian chicken breeds. Animals. 10:1441.
12. Cerolini S, Zaniboni L, Mangiagalli MG, Gliozzi TM. 2007. Effect of glycine on cryopreservation of chicken spermatozoa. Av and Poult Biol Rev. 18(2):65.
13. Cerolini S, Vasconi M, Sayed AA, Iaffaldano N, Mangiagalli MG, Pastorelli G, Moretti VM, Zaniboni L, Mosca F. 2019. Free-range rearing density for male and female Milanino chickens: Carcass yield and qualitative meat traits. J Appl Poult Res. 28:1349–1358.
14. Chalah T, Seigneurin F, Blesbois, E, Brillard JP. 1999. In vitro comparison of fowl sperm viability in ejaculates frozen by three different techniques and relationship with subsequent fertility in vivo. Cryobiology. 39:185–191.
15. Çiftci HB, Aygün A. 2018. Poultry semen cryopreservation technologies. World's Poult Sci J 74: 699-799.
16. Delany ME. 2004. Genetic variants for chick biology research: From breeds to mutants. Mech Dev. 121:1169–1177.
17. Di Iorio M, Rusco G, Iampietro R, Colonna MA, Zaniboni L, Cerolini S, Iaffaldano N. 2020a. Finding an Effective Freezing Protocol for Turkey Semen: Benefits of Ficoll as Non-Permeant Cryoprotectant and 1:4 as Dilution Rate. Animals. 10:42.
18. Di Iorio M , Rusco G, Iampietro, Maiuro L, Schiavone,Cerolini S, Iaffaldano N. 2020b.Validation of the Turkey semen cryopreservation by evaluating the effect of two diluents and the inseminating doses. Animals. 10:8-1329.
19. Donoghe AM, Wishart GJ. 2000. Storage of poultry semen. Anim Reprod Sci. 62:213–232.
20. Ehling C, Taylor U, Baulain U, Weigend S, Henning M, Rath D. 2012. Cryopreservation of semen from genetic resource chicken lines. Agric Forest Res. 62:151–158.

21. FAO DAD-IS. [accessed on 1 July 2020]. http://www.fao.org/dad-is/browse-by-country-and-species/en/.
22. FAO. 1998. Secondary guidelines for development of national farm animal genetic resources management plans. Management of small populations at risk. Rome. [accessed on 18 November 2020]. http://www.fao.org/documents/card/fr/c/ d074fc39-aca0-58ab-8024-beaf80ba6014/).
23. FAO. 2003. Community-based management of animal genetic resources. Proceedings of the workshop held in Mbabane, Swaziland 7-11 May 2001. Rome. [accessed on 19 November 2020]. www.fao.org/DOCREP/006/Y3970E/ Y3970E00.htm).
24. FAO. 2009. Preparation of national strategies and action plans for animal genetic resources. FAO Animal Production and Health Guidelines. No. 2. Rome. [accessed on 14 October 2020]. http://www.fao.org/ docrep/012/i0770e/i0770e00.htm.
25. FAO. 2012. Cryoconservation of animal genetic resources. FAO Animal Production and Health Guidelines No. 12. Rome.
26. FAO. 2015. The Second Report on the State of the World's Animal Genetic Resources for Food and Agriculture, edited by B.D. Scherf & D. Pilling. FAO Commission on Genetic Resources for Food and Agriculture Assessments. Rome. [accessed on 16 September 2020]. http://www.fao.org/3/a-i4787e/index.htmL.
27. Franzoni A, Gariglio M, Castillo A, Soglia D, Sartore S, Buccioni A, Mannelli F, Cassandro M, Cendron F, Castellini C, Mancinelli Cartoni A, Cerolini S, Sayed AA, Iaffaldano N, Di Iorio M, Marzoni M, Salvucci S, Schiavone A. 2021. Overview of Native Chicken Breeds in Italy: Small Scale Production and Marketing. Animals. 11:629.
28. Fulton JE. 2006. Avian genetic stock preservation: an industry perspective. Poult Sci. 85:227-231.
29. Garner DL, Thomas CA, Gravance CG. 1999. The effect of glycerol on the viability, mitochondrial function and acrosomal integrity of bovine spermatozoa. Reprod Dom Anim. 34: 399–404.
30. Gandini G, Oldenbroek K. 2007. Strategies for moving fom conservation to utilization. In: Utilisation and conservation of animal genetic resources. Edited by Oldenbroek K. Wageningen Academic Publishers, The Netherlands: 29-54.
31. Iaffaldano N, Manchisi A, Gambacorta M, Di Iorio M, Rosato MP. 2009. Effect of different sperm concentrations on the post-thaw viability and motility of turkey spermatozoa cryopreserved by the pellet method. Ital J Anim Sci. 8: 760–762.
32. Iaffaldano N, Romagnoli L, Manchisi A, Rosato MP. 2011. Cryopreservation of turkey semen by the pellet method: Effects of variables such as the extender, cryoprotectant concentration, cooling

time and warming temperature on sperm quality determined through principal components analysis. Theriogenology. 76:794–801.

33. Iaffaldano N, Di Iorio M, Cerolini S, Manchisi A, 2016a. Overview of turkey semen storage: Focus on Cryopreservation A review. Ann Anim Sci. 16:961–974.
34. Iaffaldano N, Di Iorio M, Miranda M, Zaniboni L, Manchisi A, Cerolini S. 2016b. Cryopreserving turkey semen in straws and nitrogen vapour using DMSO or DMA: Effects of cryoprotectant concentration, freezing rate and thawing rate on post-thaw semen quality. Br Poult Sci. 57:264–270.
35. Kim SW, Choi JS, Ko YG, Do YJ, Byun M, Park SB, Seong HH, Kim CD. 2014. Effect of N-Methylacetamide concentration on the fertility and hatchability of cryopreserved Ogye rooster semen. Korean J. Poult. Sci., 41(1): 21-27.
36. Lee, H.J., Kim, S.K., Jang, H.J., Kang, K.S., Kim, J.H., Choi, S.B., Han, J.Y. 2012. Cryopreservation of Korean Oge chicken semen using N-metylacetamide. CryoLetters. 33:427–434.
37. Leroy G, Boettcher P, Besbes B, Danchin-Burge C, Baumung R, Hiemstra SJ. 2019. Cryoconservation of Animal Genetic Resources in Europe and Two African Countries: A Gap Analysis. Divers. 11:240
38. Liptoi K, Horvath G, Gal J, Varadi E, Barna J. 2013. Preliminary results of the application of gonadal tissue transfer in various chicken breeds in the poultry gene conservation. Anim Reprod Sci. 141:86–89.
39. Long JA. 2006. Avian semen cryopreservation: What are the biological challenges?. Poult Sci. 85:232–236.
40. Madeddu M, Mosca F, Sayed AA, Zaniboni L, Mangiagalli MG, Colombo E, Cerolini S. 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. Anim Reprod Sci. 171:58–64.
41. Marzoni M. 2008. Inseminazione artificiale. In: Avicoltura e Coniglicoltura. Editors Cerolini S., Marzoni M., Romboli I., Schiavone A., Zaniboni L, Le Point Veterinaire Italie, Milano: 125-138.
42. Meuwissen T. 2017. Operation of conservation schemes. In: Utilisation and conservation of animal genetic resources. Edited by Oldenbroek K. Wageningen Academic Publishers, The Netherlands: 167-193.
43. Miranda M, Kulíková B, Vašíček J, Olexiková L, Iaffaldano N, Chrenek P. 2018. Effect of cryoprotectants and thawing temperatures on chicken sperm quality. Reprod Domest Anim. 53:93–100.

44. Mosca F, Madeddu M, Sayed AA, Zaniboni L, Iaffaldano N, Cerolini S. 2016a. Combined effect of permeant and non-permeant cryoprotectants on the quality of frozen/thawed chicken sperm. Cryobiology 73:343–347.
45. Mosca F, Madeddu M, Sayed AA, Zaniboni L, Iaffaldano N, Cerolini S. 2016b. Data on the positive synergic actionof dimethylacetamide and trehalose on quality of cryopreserved chicken sperm. Data Brief. 9:1118–1121.
46. Mosca F, Zaniboni L, Sayed AA, Madeddu M, Iaffaldano N, Cerolini S. 2019. Effect of dimethylacetamide and N-methylacetamide on the quality and fertility of frozen/thawed chicken semen. J Poult Sci. 98:6071–6077.
47. Mosca F, Zaniboni L, Sayed AA, Iaffaldano N, Soglia D, Schiavone A, Cerolini S. 2020. Effect of N-Methylacetamide concentration and thawing rate on chicken sperm quality after cryopreservation. Animals. 10(5): 824.
48. Nakamura Y, Tasai M, Takeda K, Nirasawa K, Tagami T. 2013. Production of Functional Gametes from Cryopreserved Primordial Germ Cells of the Japanese Quail. J Reprod Dev. 59:580–587.
49. Nakamura Y. 2016. Poultry genetic resource conservation using primordial germ cells. J Reprod Develop. 62:431–437.
50. Nandi S, Whyte J, Taylor L, Sherman A, Nair V, Kaiser P, McGrew MJ. 2016. Cryopreservation of specialized chicken lines using cultured primordial germ cells. Poult Sci. 95:1905–1911.
51. Ozdemir D, Ozdemir ED, De Marchi M, Cassandro M. 2013. Conservation of local Turkish and Italian chicken breeds: a case study. Ital J Anim Sci. 12:313-319.
52. Paiva SR, McManus CM, Blackburn H. 2016. Conservation of animal genetic resources – A new tact. Livest Sci. 193: 32-38.
53. Patterson DL, Silversides FG. 2003. “Farm Animal Genetic Resource Conservation. Why and how?”. [accessed on 16 December 2020]. http://www.cfagrf.com/FarmAnimalGenteticResourceConservation WhyandHow.htm.
54. Pranay Kumar, K., Swathi, B., Shanmugam, M. 2018. Cryopreservation of rooster semen using N-methylacetamide as cryoprotective agent. Int. J. Agric. Sci. 10, 5123–5126.
55. Prentice JR, Anzar M. 2010. Cryopreservation of mammalian oocyte for conservation of animal genetics. Vet Med Int. 2011:146-405.
56. Santiago-Moreno J, Castaño C, Toledano-Díaz A, Coloma MA, López- Sebastián A, Prieto MT, Campo JL. 2011. Semen cryopreservation for the creation of a Spanish poultry breeds cryobank: Optimization of freezing rate and equilibration time. Poult Sci. 90:2047–2053.

57. Sasaki K, Tatsumi T, Niinomi T, Imai T, Naito M, Tajima A, Nishi Y. 2010. A method for cryopreserving semen from Yakido roosters using N-Methylacetamide as a cryoprotective agent. J Poult Sci. 47: 297–301.
58. Shanmugam, M., Pranay Kumar, K., Mahapatra, R.K., Anand Laxmi, N. 2018. Effect of different cryoprotectants on post-thaw semen parameters and fertility in Nicobari chicken. Indian J. Poult. Sci. 53, 208–211
59. Silversides FG, Purdy PH, Blackburn HD. 2012. Comparative costs of programmes to conserve chicken genetic variation based on maintaining living populations or storing cryopreserved material. Br Poult Sci. 53(5):599-607.
60. Soglia D, Sartore S, Lasagna E, Castellini C, Cendron F, Cassandro M, Marzoni M, Iaffaldano I, Buccioni A, Dabbou S, Castillo A, Maione S, Bianchi S, Profiti M, Sacchi P, Cerolini S, Schiavone S. 2021. Italian poultry biodiversity of 17 Autochthonous Chicken Breeds and their extinction risk status. Frontiers in Genetics, section Livestock Genomics, in press.
61. Swain JE, Smith GD. 2010. Cryoprotectants, in: CHIAN, R. C. & QUINN, P. (Eds) Fertility Cryopreservation, Vol. 4, pp. 24– 38 (New York, Cambridge University Press).
62. Szalay I. 2004. A régi magyar baromfifajták tenyésztése és génvédelme a KÁTKI-ban. [Breeding and conservation of old Hungarian poultry breeds in KATKI. In Hungarian]. A Baromfi. 7(3):22–25.
63. Sztan N, Lazar B, Bodzsar N, Vegi B, Liptoi K, Pain B, Varkonyi EP. 2017. Successful chimera production in the Hungarian goose (*Anser anser domestica*) by intracardiac injection of blastodermal cells in 3-day-old embryos. Reprod Fert Develop. 29:2206–2216.
64. Thélie A, Bailliard A, Seigneurin F, Zerjal T, Tixier-Boichard M, Blesbois E. 2019. Chicken semen cryopreservation and use for the restoration of rare genetic resources. Poult Sci. 98:447–455.
65. Tselutin K, Seigneurin F, Blesbois E. 1999. Comparison of cryoprotectants and method of cryopreservation of fowl spermatozoa. Poult Sci. 78:586–590.
66. Woelders H, Zuidberg CA, Hiemstra SJ. 2006. Animal genetic resources conservation in the Netherlands and Europe: Poultry perspective. Poult Sci. 85:216–222.
67. Zaniboni L, Cassinelli C, Mangiagalli MG, Gliozzi TM, Cerolini S. 2014. Pellet cryopreservation for chicken semen: Effects of sperm working concentration, cryoprotectant concentration, and equilibration time during in vitro processing. Theriogenology. 82: 251−258.
68. Zaniboni L, Madeddu M, Mosca M, Abdel Sayed Ahmad, Marelli SP, Di Iorio M, Iaffaldano N, Cerolini S. 2021. Concentration dependent effect of dimethylacetamide and N-methylacetamide

on the quality and fertility of cryopreserved chicken semen. Cryobiology, submitted for publication.

69. Zanon A, Sabbioni A. 2001. Identificazione e salvaguardia genetica delle razze avicole Italiane. Ann Med Vet. 21:117–134.

STUDY 2

Biology 2022

(doi: 10.3390/biology11050642)

biology

MDPI

Article

The Effect of Semen Cryopreservation Process on Metabolomic Profiles of Turkey Sperm as Assessed by NMR Analysis

Gianluca Paventi [1], Michele Di Iorio [2], Giusy Rusco [2], Anatoly P. Sobolev [3], Silvia Cerolini [4], Emanuele Antenucci [2], Mattia Spano [5], Luisa Mannina [5] and Nicolaia Iaffaldano [2,*]

[1] Department of Medicine and Health Sciences "Vincenzo Tiberio", University of Molise, 86100 Campobasso, Italy; paventi@unimol.it
[2] Department of Agricultural, Environmental and Food Sciences, University of Molise, 86100 Campobasso, Italy; michele.diiorio@unimol.it (M.D.I.); giusy.rusco@unimol.it (G.R.); e.antenucci@studenti.unimol.it (E.A.)
[3] Institute for Biological Systems, Magnetic Resonance Laboratory "Segre-Capitani", CNR, 00015 Monterotondo, Italy; anatoly.sobolev@cnr.it
[4] Department of Veterinary Medicine, University of Milano, 26900 Lodi, Italy; silvia.cerolini@unimi.it
[5] Department of Chemistry and Technology of Drugs, Sapienza University of Rome, 00185 Rome, Italy; mattia.spano@uniroma1.it (M.S.); luisa.mannina@uniroma1.it (L.M.)
* Correspondence: nicolaia@unimol.it

Simple Summary: Semen cryobanking is a valuable tool for preserving the genetic resources of a wide range of species, providing the opportunituy to preserve representative samples and reconstruct the population or diversity. However, in avian species, the freezing–thawing process results in a sharp reduction in sperm quality and consequently fertility. This is mainly due to the lack of knowledge about the molecular basis of the cryopreservation process, especially in more sensitive species such as turkey. Thus, in this study, we took advantage of NMR technology to assess the changes in metabolic profile occurring in turkey sperm cryopreservation, which were correlated with sperm qualitative parameters measured in both fresh and frozen–thawed samples. Hence, the results reported here depict a clearer scenario about the changes in the levels of amino acids, other water-soluble compounds, and lipids resulting from the freezing–thawing process. Moreover, a wide discussion about the possible pathway affected by cryopreservation is provided. Therefore, this study allows us to: *(i)* identify biological markers related to the sperm freezability of male turkey donators; *(ii)* suggest a supplementation of specific metabolites in the diet or in the freezing medium in order to obtain spermatozoa abler to withstand the freezing process.

Abstract: Semen cryopreservation represents the main tool for preservation of biodiversity; however, in avian species, the freezing–thawing process results in a sharp reduction in sperm quality and consequently fertility. Thus, to gain a first insight into the molecular basis of the cryopreservation of turkey sperm, the NMR-assessed metabolite profiles of fresh and frozen–thawed samples were herein investigated and compared with sperm qualitative parameters. Cryopreservation decreased the sperm viability, mobility, and osmotic tolerance of frozen–thawed samples. This decrease in sperm quality was associated with the variation in the levels of some metabolites in both aqueous and lipid sperm extracts, as investigated by NMR analysis. Higher amounts of the amino acids Ala, Ile, Leu, Phe, Tyr, and Val were found in fresh than in frozen–thawed sperm; on the contrary, Gly content increased after cryopreservation. A positive correlation ($p < 0.01$) between the amino acid levels and all qualitative parameters was found, except in the case of Gly, the levels of which were negatively correlated ($p < 0.01$) with sperm quality. Other water-soluble compounds, namely formate, lactate, AMP, creatine, and carnitine, turned out to be present at higher concentrations in fresh sperm, whereas cryopreserved samples showed increased levels of citrate and acetyl-carnitine. Frozen–thawed sperm also showed decreases in cholesterol and polyunsaturated fatty acids, whereas saturated fatty acids were found to be higher in cryopreserved than in fresh sperm. Interestingly, lactate, carnitine ($p < 0.01$), AMP, creatine, cholesterol, and phosphatidylcholine ($p < 0.05$) levels were positively correlated with all sperm quality parameters, whereas citrate ($p < 0.01$), fumarate, acetyl-carnitine, and saturated fatty acids ($p < 0.05$) showed negative correlations. A detailed discussion aimed at explaining these correlations in the sperm cell context is provided, returning a clearer scenario of metabolic changes occurring in turkey sperm cryopreservation.

check for updates

Citation: Paventi, G.; Di Iorio, M.; Rusco, G.; Sobolev, A.P.; Cerolini, S.; Antenucci, E.; Spano, M.; Mannina, L.; Iaffaldano, N. The Effect of Semen Cryopreservation Process on Metabolomic Profiles of Turkey Sperm as Assessed by NMR Analysis. *Biology* **2022**, *11*, 642. https://doi.org/10.3390/biology11050642

Academic Editors: Pilar Santolaria Blasco, Rosaura Pérez-Pé and Jessica Rickard

Received: 11 March 2022
Accepted: 20 April 2022
Published: 22 April 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Conservation of genetic variability in domestic animal species is a task for the sustainable production of human food resources, as well as for land management and, more importantly, the preservation of biodiversity (Blesbois et al., 2007; Partyka and Niżański 2022). Currently, despite the development of other innovative strategies, such as gonadal tissue allotransplantation (Liptoi et al.,2020) and diploid primordial germ cell methodologies (Santiago-Moreno and Blesbois 2011), semen cryopreservation still remains the most effective method to store reproductive cells for the *ex situ* management of genetic diversity in birds (Blesbois 2011; Kowalczyk and Łukaszewicz 2015; Thélie et al.,2019). Thus, the growth of this *ex situ in vitro* strategy, as a support to the *in vivo* strategy, is becoming more advanced. In this regard, the constitution of cryobanks for genetic resources would offer a crucial link between both strategies, leading to the improvement of conservation programs efficiency (Prentice et al., 2011; Iaffaldano et al., 2021).

However, the main challenge for the creation of a poultry semen cryobank is the achievement of a successful freezing protocol (Iaffaldano et al., 2021). In relation to this, it is known that the semen cryopreservation process causes a loss of sperm membrane integrity in poultry (Partyka and Niżański 2022; Blesbois 2011; Iaffaldano et al., 2016). Thus, the improvement of sperm cryosurvival and increased fertility in artificial insemination (AI) with frozen–thawed sperm continues to be the focus in semen cryobanking in avian species. This is even more evident in turkey species, the semen of which has been proven to be more sensitive to cooling and freezing–thawing damages than chicken sperm (Blanco et al., 2000, 2008; Blesbois 2007; Iaffaldano et al., 2011, 2016a,2016b; Izanloo et al., 2021). Though chicken and turkey sperm share the same morphology (as a filiform shape, a long tail and a condensed nucleus (Blesbois et al., 2005), turkey sperm presents a high cholesterol/phospholipid ratio, resulting in low membrane fluidity and permeability (Blesbois et al., 2005); moreover, it has a low osmotic resistance at hypo-osmotic conditions (Blanco et al., 2008).

For these reasons, over the last few decades, several studies have been performed, in which different factors involved in sperm cryosurvivability were taken into consideration, to identify an efficient freezing procedure for both chicken and turkey semen (Iaffaldano et al., 2009,2011; Di Iorio et al., 2020a,2020b; Mosca et al., 2016, 2019,2020; Zaniboni et al.,2006). However, despite the encouraging results obtained so far, there is still a gap of knowledge about the biological bases involved in the cryopreservation process. This limits the development of a freezing procedure that results in fertilization rates closer to those obtained with fresh semen. In addition, more efficient semen cryopreservation, besides ensuring the conservation of genetic resources in a gene bank, could provide practical advantages to the turkey industry, since commercial farms are completely dependent on AI to obtain fertile eggs (Iaffaldano et al., 201). The avian sperm membrane contains more

polyunsaturated fatty acids (PUFAs) than that of mammals, and it has lower protein content, a lower cholesterol/phospholipid ratio, and greater overall fluidity at physiological temperatures (Parks et al., 1992; Cerolini et al., 2006).

Specific biological and biophysical factors, such as membrane permeability, lipid composition, and membrane fluidity, can affect the ability of poultry spermatozoa to limit damages caused by the cryopreservation procedure (Blesbois et al., 2008; Long et al.,2014).

In this regard, it was reported that the freezing–thawing procedure for avian spermatozoa induces a membrane rigidifying process that is accompanied by a dramatic and proportional decrease in the cholesterol/phospholipid ratio. Moreover, this effect is different from species to species; thus, it could behave as an indicator of between-species freezability (Blesbois et al., 2005,2008). Accordingly, it was also observed that the ratio of lipids in the sperm membrane determines the overall fluidity of the membrane and impacts the ability of sperm to remain viable during the cryopreservation process in chicken (Blesbois et al., 2005; Mussa et al., 2021) as well as in mammals (Mandal et al., 2014). This is consistent with previous findings in turkey showing that lipids are involved in vital aspects of sperm metabolism and functions (Zaniboni et al., 2009).

Besides lipids, other factors contribute to defining membrane fluidity in poultry (Long 2006), such as the nature and the level of insertion of the proteins in the membrane lipid bilayer (Shinitzky et al., 1982). In addition, amino acids could play a role in avian sperm function as shown for mammals: some amino acids participate in many metabolic processes involved in motility, acrosome reaction, and capacitation of human and other mammalian spermatozoa (Vickram et al., 2012; Cheah and Yang 2011). Amino acids also have antioxidant properties able to protect sperm cells from cold shock (Atessahin et al., 2008; Ugur et al., 2020); consistently, plasma amino acids seem to play a role in chicken sperm cryoresistance (Santiago-Moreno et al., 2019). In mammals, it has been demonstrated that amino acids act at the extracellular level and improve sperm motility, acrosome integrity, and fertilizing potential after the freezing–thawing process (Renard et al.,1996; Li et al.,2003; Khlifaoui et al., 2005; Bucak et al., 2009; Moradi et al., 2022). It has also been reported that L-carnitine is involved in sperm energy metabolism, promoting sperm motility and maturation and the spermatogenic process (Jeulin C. et al., 1996; Agarwal et al., 2004). Thus, its supplementation was proposed to increase both kinetics and morphological characteristics of sperm (Stradaioli et al., 2004). On the contrary, less information is known about these aspects in avian species, especially in turkey. The sperm metabolite profile, which includes lipids, amino acids, and other watersoluble compounds, appears to be the main factor that affects sperm resilience the cryopreservation process, which in turn determines the fertilizing ability of sperm (Yu et al.,2022)

Even now, there is a complete lack of knowledge about the turkey sperm metabolite profile. The only investigation on metabolite profile of fresh turkey sperm reported in literature was our previous study, in which changes in metabolite levels occurring in male reproductive ageing were measured by using nuclear magnetic resonance (NMR) (Iaffaldano et al., 2018). Currently, there is still no scientific evidence in the literature about the metabolite profile changes during the semen cryopreservation process. Thus, by detecting and simultaneously quantifying a wide range of metabolites with a high analytical precision, NMR represents a valuable tool for better understanding the biological bases of the turkey semen cryopreservation process.

In this study, deeper insight into the metabolite profile changes occurring in turkey sperm cryopreservation was obtained by assessing via NMR a relevant number of metabolites in both fresh and frozen–thawed samples. The changes in metabolite levels were further correlated to sperm quality variations after thawing and are herein discussed from a metabolic point of view.

2. Materials and Methods

2.1. Chemicals

The fluorescent dyes SYBR-14 and propidium iodide (PI) used were those provided in the LIVE/DEAD Sperm Viability kit (Invitrogen^{TM} by Thermo Fisher Scientific, Waltham, MA, USA). All the other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals and Semen Treatment

Hybrid Large White turkey males from a private breeding group (Agricola Santo Stefano of Amadori's group, Canzano, TE, Italy) were used. Animals were housed there when they were 32 weeks old. They were maintained under standard management conditions and photostimulated on a daily basis with a 14L:10D photoperiod. The toms were kept in groups of 8–10 in floor pens. Feed and water were provided ad libitum. Toms were trained for semen collection by abdominal massage two times a week.

Semen was collected from 32 weeks of age males by abdominal massage. Ejaculates were pooled, with each pool originating from a minimum of 9 to a maximum of 12 males,

and thoroughly mixed in order to reach at least 4 mL of semen/pool. In total, 5 pools of semen were used in this study.

2.3. Cryopreservation Process

Semen was cryopreserved by the pellet method Iaffaldano et al., 2011. In brief, semen samples were diluted (1:4) in Tselutin extender (Tselutin et al., 1995). The diluted semen was cooled at 4°C for 60

min, and then 8% (*v/v*; 0.860 M) of dimethylacetamide (DMA) was added as cryoprotectant. The semen was gently inverted and equilibrated for 5 min at 4°C. Volumes of 80 µL of semen were plunged drop by drop directly into liquid nitrogen to form spheres of frozen semen (pellets). The pellets were rapidly placed in 2 mL polypropylene cryovials (Cryo.sTM; Greiner Bio-One, Monroe, NC, USA) previously cooled by immersion in liquid nitrogen (3–4 pellets/cryovial) and then stored in a liquid nitrogen tank until analysis. After two weeks, the pellets were warmed by immersing the cryovials in a water bath at 75°C for 12 s.

2.4. Semen Quality Evaluation

Mobility, viability and sperm osmotic tolerance were assessed on both freshly diluted and frozen–thawed samples. To this end, each sperm sample was divided in two aliquots: one to be immediately assessed (fresh), and the other to be subjected to freezing–thawing process.

Sperm mobility was evaluated using the Sperm Motility Test (SMT) according to the Accudenz® procedure (Accurate Chemical & Scientific Corp., Westbury, NY, USA) following the procedure described by Iaffaldano et al. 2011, 2018. This procedure is based on the ability of the spermatozoa with a forward progressive motility to penetrate a 4% Accudenz® layer. Semen was diluted to 1.0 $x10^9$ as previously described Iaffaldano et al. 2018. A drop of 60 µL from each sperm suspension was superimposed onto 600 µL of 4% (*w/v*) Accudenz® solution in a semimicro polystyrene disposable cuvette. Cuvettes were incubated for 5 min in a 41°C water bath, and absorbance was measured in a spectrophotometer at 550 nm after 60 s. The sperm motility was expressed by values of optical density (O.D.).

Sperm viability was measured using the Invitrogen™ LIVE/DEAD sperm viability kit according to the procedure set up by Iaffaldano et al 2011. Aliquots of 5 µL semen were diluted in 39 µL of Tselutin diluent containing 1 µL of SYBR-14 (diluted 1:100 into dimethylsulfoxide). Samples were incubated for 10 min at 38°C. Then, 5 µL of propidium iodide (PI; dissolved 1:100 in PBS) was added, and the samples were further incubated at 38°C for 5 min. The assessment of viable/nonviable spermatozoa was performed using fluorescence microscopy (blue excitation filter λ = 488 nm; ×100 oil immersion objective; magnification × 400). Viable sperm cells were stained green by SYBR-14, whereas dead cells were stained in red by PI. A minimum of 200 spermatozoa for each sample were counted. Percentages of viable spermatozoa were determined as the ratio: green cells/(green cells + red cells) ×100.

Sperm osmotic tolerance (SOT) was assessed using a hypo-osmotic swelling test (HOST) (Iaffaldano 2011,2016). Five microliters of semen were added to 80 µL of distilled H_2O and then stained with SYBR-14/PI and read as described above for sperm viability.

This test is effective for assessing the percentage of viable spermatozoa that are capable of withstanding hypo-osmotic stress *in vitro*. Under hypo-osmotic conditions, viable thawed spermatozoa with intact membranes fluoresce green (SYBR) and exclude PI. Conversely, damaged membranes permit the passage of PI, staining spermatozoa that have lost their functional integrity red.

2.5. NMR Measurements

2.5.1. Sample Preparation

The Bligh–Dyer (Bligh and Dyer 1959) method was used to extract and separate water-soluble and liposoluble metabolites from semen samples following the procedure previously reported (Ingallina et al., 2020a).

Before NMR analysis, fresh and frozen semen was diluted at the same concentration and then centrifuged at 1500 rpm for 15 min to remove both diluent and seminal plasma. A chloroform/methanol (2:1, *v/v*) mixture was added to the sperm pellets, and the samples were homogenized with a vortex mixer for 60 s before adding distilled water in the proportion of 1:18:4 (spermatozoa–chloroform/methanol–water). The homogenate was centrifuged at a speed of 4000 rpm for 20 min at 5°C. The liquid chloroform and water/methanol phases were separated and dried under vacuum in a rotary evaporator. The dried residues were dissolved into 0.75 mL of CDCl3/CD3OD (2:3 *v/v*) or 0.75 mL of D2O phosphate buffer (400 mM, pD = 7).

2.5.2. NMR Spectra

The NMR spectra of aqueous and organic extracts were recorded at 27°C on a Bruker AVANCE 600 NMR spectrometer operating at the proton frequency of 600.13 MHz and equipped with a Bruker multinuclear z-gradient inverse probe head capable of producing gradients in the z-direction with a strength of 55 G/cm. ^{1}H spectra were referenced to methyl group signals of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP,= δ 0.00 ppm) in D2O and to the residual CHD2 signal of methanol (set to 3.31 ppm) in CDCl3/CD3OD mixture (Ingallina et al., 2020b). 1H spectra of aqueous extracts were acquired by coadding 512 transients with a recycle delay of 3 s. The residual HDO signal was suppressed using a standard Bruker presaturation sequence zgpr. The experiment was carried out by using a 45° pulse of 7.25 μs and 32,000 data points. ^{1}H spectra of CDCl3/CD3OD extracts were obtained using the following parameters: 256 transients, 32,000 data points, a recycle delay of 3 s, and a 90° pulse of 10 μs. The ^{1}H spectra were Fourier transformed using an exponential multiplication function with a line broadening factor of 0.3 Hz, and manual phase correction and baseline correction were applied.

2.5.3. Measurement of the Metabolic Content in Aqueous Extract

The intensity of 21 ^{1}H resonances due to water-soluble assigned metabolites (see Table 1) was measured with respect to the intensity of a TSP signal used as internal standard and normalized to 100.

2.5.4. Measurement of the Metabolic Content in Organic Extracts

The integrals of 8 ^{1}H resonances due to assigned liposoluble metabolites were measured and used to obtain the normalized integrals, see Table 1. All the integrals were normalized with respect to the integrals of α-CH_2 groups of all fatty acid chains at 2.31 ppm set to 100%. The molar percentages of lipids were calculated taking into account the number of equivalent protons corresponding to a specific resonance. The resonances due to the CH_3 of cholesterol (0.74 ppm), all allylic protons (2.08 ppm), α-CH_2 groups of all fatty acid (2.31 ppm), CH_2 diallylic protons of DUFA, (2.81 ppm), CH_2 diallylic protons of PUFA (2.88 ppm), CH_2N of PE (3.21 ppm), $(CH_3)_3N^+$ of PC (3.28 ppm), and CH (double bond) proton of SMN (5.76 ppm) were integrated. The molar percentage of all saturated fatty chains (SFA) was calculated as 100 UFA, where UFA was calculated using the all-allylic-protons signal at 2.08 ppm.

2.6. Statistical analysis

Sperm qualitative parameters and metabolite levels determined by NMR analysis measured in fresh and frozen–thawed sperm were compared by paired-samples t-test (threshold at $p < 0.05$). Correlations between sperm variables and NMR-identified metabolites were assessed through Pearson's correlation coefficients, setting significance thresholds at the $p < 0.05$ level (one-tailed) and $p < 0.01$ levels (two-tailed). All statistical tests were performed using the software package SPSS v23.0 (SPSS, Chicago, IL, USA).

TABLE 1- Metabolites identified and quantified by NMR in fresh and frozen sperm of turkey males at 32 weeks of age.

Metabolite, ^{1}H chemical shift (ppm)	fresh	frozen	
	Water extract		
Amino acids	**mol % (*n* = 5)**	**mol % (*n* = 5)**	***p-value***
Ala (1.48)	**0.178 ± 0.007** [a]	**0.113 ± 0.007** [b]	***0.001***
Asp (2.83)	0.239 ± 0.022 [a]	0,221 ± 0.005 [a]	*0.466*
Gln (2.45)	1.614 ± 0.314 [a]	1.468 ± 0.197 [a]	*0.412*
Glu (2.07)	66.547 ± 0.391 [a]	66.956 ± 0.761 [a]	*0.725*
Gly (3.57)	**4.648 ± 0.043** [a]	**4.951 ± 0.073** [b]	***0.001***
Ile (1.02)	**0.015 ± 0.001** [b]	**0.009 ± 0.001** [b]	***0.009***
Leu (0.96)	**0.054 ± 0.003** [a]	**0.026 ± 0.001** [b]	***0.003***
Phe (7.43)	**0.022 ± 0.001** [a]	**0.017 ± 0.001** [b]	***0.030***

Tyr (6.92)	**0.048 ± 0.002** [a]	**0.020 ± 0.002** [b]	***0.001***
Val (0.99)	**0.038 ± 0.002** [a]	**0.019 ± 0.002** [b]	***0.002***
Organic acids			
Acetate (1.93)	0.486 ± 0.064 [a]	0.353 ± 0.087 [a]	*0.403*
Citrate (2.57)	**0.089 ± 0.006** [b]	**0.125 ± 0.006** [a]	***0.039***
Formate (8.46)	**0.039 ± 0.003** [a]	**0.019 ± 0.004** [b]	***0.002***
Fumarate (6.53)	0.032 ± 0.003 [a]	0.040 ± 0.002 [a]	*0.072*
Lactate (1.33)	**1.362 ± 0.072** [a]	**0.884 ± 0.059** [b]	***0.001***
Other compounds			
Ac-carnitine (3.20)	**0.037 ± 0.002** [a]	**0.045 ± 0.002** [b]	***0.006***
AMP (8.28)	**0.146 ± 0.007** [a]	**0.115 ± 0.008** [b]	***0.005***
Carnitine (3.24)	**0.080 ± 0.005** [a]	**0.037 ± 0.002** [b]	***0.002***
Creatine (3.94)	**1.828 ± 0.136** [a]	**1.431 ± 0.071** [b]	***0.028***
Glucose (3.26 and 5.25)*	16.445 ± 0.437 [a]	17.085 ± 0.592 [a]	*0.504*
Myo-inositol (3.65)	6.054 ± 0.039 [a]	6.067 ± 0.205 [a]	*0.949*
Lipid Extract			
	mol % (n = 3)	**mol % (n = 3)**	
CHO (0.74)	**9.587 ± 0.348** [a]	**6.944 ± 0.533** [b]	***0.036***
SFA	**38.001 ± 1.430** [b]	**43.088 ± 1.032** [a]	***0.006***
DUFA (2.81)	4.200 ± 0.136 [a]	3.857 ± 0.356 [a]	*0.293*
UFA (2.08)	**62.000 ± 1.430** [a]	**56.912 ± 1.032** [b]	***0.006***
PUFA (2.86)	**36.793 ± 0.561** [a]	**34.665 ± 0.633** [b]	***0.027***
PC (3.28)	24.703 ± 0.760 [a]	19.667 ± 0.612 [a]	*0.081*
PE (3.21)	14.107 ± 0.152 [a]	11.989 ± 0.611 [a]	*0.072*
SMN (5.76)	6.990 ± 0.239 [a]	6.200 ± 0.577 [a]	*0.167*

[a,b] Different superscript letters within the same row indicate significant differences ($p < 0.05$). Abbreviations: Accarnitine: acylcarnitine; AMP: adenosine monophosphate; CHO: cholesterol; SFA: total content of saturated fatty acids; DUFA: diunsaturated fatty acids; UFA: total content of unsaturated fatty acids; PUFA: polyunsaturated fatty acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; SMN: sphingomyelin.

3. Results

3.1. Sperm quality

The sperm quality parameters recorded in freshly collected and frozen–thawed sperm samples are provided in Figure 1. The cryopreservation process severely affected all of the measured qualitative parameters. A significant reduction was found in sperm viability, as assessed by fluorescence microscopy (inset Figure 1a); these values, in fact, were lower by about 50% in frozen-thawed than in fresh samples (Figure 1a). A similarly remarkable decrease was observed in the sperm mobility of cryopreserved sperm (Figure 1b). The sperm osmotic tolerance also suffered from a dramatic decrease, with the values measured in cryopreserved sperm 40% lower than those measured in fresh samples (Figure 1c).

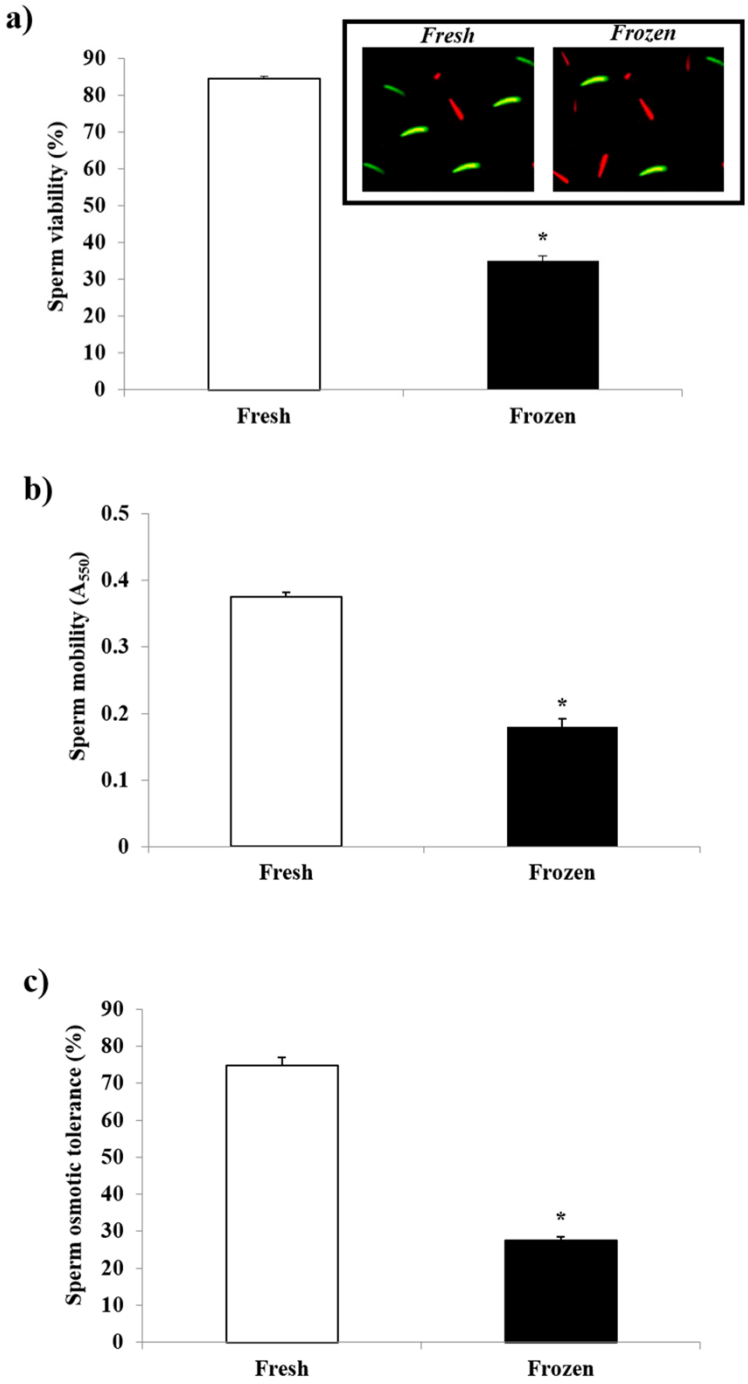


Figure 1. Effect of the cryopreservation process on the (**a**) viability, (**b**) mobility, and (**c**) osmotic tolerance of turkey sperm. Mean values ± SE (n = 5) of sperm qualitative parameters recorded for either fresh or frozen–thawed turkey sperm were reported. (**a**) Viability values, expressed as %, were measured by means of a dual staining technique (as shown in inset) using the stains SYBR-14 (green, viable cells) and PI (red, dead cells). (**b**) Mobility values, expressed as Abs 550 nm, were measured by the sperm motility test. (**c**) Sperm osmotic tolerance (SOT) was assessed by the hypo-osmotic H_2O test. For further details, see Methods section. * = $p < 0.05$.

3.2. NMR Analysis

To obtain a picture of the metabolite changes due to cryopreservation, water and lipid soluble components in turkey sperm were identified in NMR spectra using the same NMR

experimental conditions and assignments from our previous study (Iaffaldano et al., 2018). All identified metabolites quantified for both fresh and cryopreserved samples are reported in Table 1. The identified water-soluble metabolites were the amino acids alanine (Ala), isoleucine (Ile), phenylalanine (Phe), leucine (Leu), valine (Val), aspartic acid (Asp), glycine (Gly), tyrosine (Tyr), glutamate (Glu) and glutamine (Gln) and other water-soluble metabolites such as lactate, acetate, citrate, creatine, acetyl-carnitine (ac-carnitine), carnitine, glucose, myo-inositol, fumaric acid, formic acid, and adenosine monophosphate (AMP).Compared with fresh samples, frozen–thawed sperm showed decreased content of Ala, Ile, Leu, Tyr, Val ($p < 0.01$), and Phe ($p < 0.05$), whereas Gly levels proved to increase in cryopreserved samples. No statistically significant differences were found for Asp, Glu, or Gln.

Moreover, significant decreases ($p < 0.01$) in formate, lactate, AMP, carnitine, and creatine levels were found in frozen–thawed sperm, which also showed increased values of citrate ($p < 0.05$) and ac-carnitine ($p < 0.01$). No significant differences were observed between fresh and frozen sperm for acetate, fumarate, glucose, or *myo*-inositol content.

In lipid extract, cholesterol (CHO), the total content of all unsaturated fatty acids (UFA), diunsaturated fatty acids (DUFA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SMN), polyunsaturated fatty acids (PUFA), and the total content of saturated fatty acids (SFA) were identified. Significantly higher values of CHO, PUFA ($p < 0.05$), and UFA (0.01) were recorded in fresh than in frozen–thawed sperm, whereas a higher value of SFA was observed in the latter samples ($p < 0.01$). No significant differences between fresh and frozen–thawed samples were scored for other lipids identified.

3.3. Correlation

In order to study the correlations between sperm quality parameters and the amounts of the different metabolites, Pearson correlation coefficients were calculated (Table 2).

Table 2 - Pearson correlations between sperm qualitative parameters and metabolites identified in fresh and frozen-thawed sperm of turkey males at 32 weeks of age.

Metabolite	Sperm variables		
	Mobility	**Viability**	**Osmotic tolerance**
Ala	0.867**	0,930**	0.902**
Gly	-0.770**	-0.771	-0.802**
Ile	0.818**	0.861**	0.908**
Leu	0.915**	0.942**	0.962**
Phe	0.683*	0.784**	0.781**
Tyr	0.915**	0.969**	0.972**
Val	0.871**	0.937**	0.947**
Citrate	-0.874**	-0.833**	-0.815**
Formate	0.764*	0.854**	0.811**
Fumarate	-0.723*		-0.659*
Lactate	0.806**	0.887**	0.830**
Ac-carnitine	-0.740*	-0.695*	-0.689*

AMP	0.653*	0.728*	0.708*
Carnitine	0.923**	0.925**	0.957**
Creatine		0.673*	0.697*
CHO	0.907*	0.884*	0.876*
SFA	-0.865*		-0.868*
UFA	0.865*		0.868*
PC	0.969**	0.882*	0.884*
PE	0.851*	0.870*	

Pearson correlation coefficients were calculated for the sperm qualitative parameters (Figure 1) versus metabolites detected by NMR (Table 1). Only significant correlation values * at the 0.05 level and ** at the 0.01 level are reported. Abbreviations:Ac-carnitine: acylcarnitine; AMP: adenosine monophosphate; CHO: cholesterol; SFA: total content of saturated fatty acids; UFA: total content of unsaturated fatty acids; PC: phosphatidylcholine; PE: phosphatidylethanolamine.

Sperm mobility, viability, and osmotic tolerance were positively correlated with Ala, Ile, Leu, Phe, Tyr, Val, formate, lactate, carnitine ($p < 0.01$), AMP, CHO, and PC ($p < 0.05$). The content of creatine was found to be positively correlated with sperm vitality and osmotic tolerance ($p < 0.05$). UFA levels correlated positively with sperm motility and osmotic tolerance ($p < 0.05$), and PE content showed positive correlations with sperm motility and sperm viability ($p < 0.05$). On the contrary, negative correlations with all qualitative parameters were found for Gly, citrate ($p < 0.01$), and ac-carnitine ($p < 0.05$), and both fumarate and SFA ($p < 0.05$) were negatively correlated with sperm motility and sperm osmotic tolerance.

4. Discussion

Results obtained by sperm quality analysis showed that the freezing–thawing process caused significant reductions in sperm motility, viability, and osmotic tolerance. These findings were in accordance with previous reports on avian sperm (Iaffaldano et al., 2011, 2016; Blesbois et al., 2005; Lemoine et al., 2011). In particular, the post-thaw semen quality measured in this study was similar to that observed in a previous paper (Iaffaldano et al.,2011): after thawing, in fact, the returned recovery rates (value found in cryopreserved semen/value found in the fresh semen × 100) were about 40%, 48%, and 37% for sperm viability, mobility, and osmotic tolerance, respectively.

It is well known that the semen cryopreservation process imposes numerous stresses not only on the physical features of sperm but on its chemical composition, which in turn is essential for sperm function, as in the case of energy metabolism, which is known to be absolutely crucial in supporting sperm motility. Thus, the high sensitivity of turkey sperm to the cryopreservation process is assumed to be a consequence of the sperm metabolic profile of this bird (also see Iaffaldano et al., 2016). In particular, the first pieces of evidence about changes in lipid content, cholesterol/phospholipid ratios, and glycoconjugate and ATP content as a result of semen cryopreservation process have been already reported (Blesbois et al., 2005; Long et al., 2006; Słowińska et al., 2018). However, a more exhaustive picture of the sperm metabolic profile before

and after cryopreservation is still lacking. Therefore, in this study, by taking advantage of NMR technique, a relevant number of metabolites were assessed in both fresh and frozen–thawed spermatozoa in order to correlate their levels to sperm quality variations.

Data obtained by NMR analysis are discussed separately for each class of compounds. *Amino Acids*. We observed a general decrease in amino acid levels in frozen–thawed sperm, with statistically significant differences for Ala, Ile, Leu, Phe, Tyr, and Val. Hence, we hypothesize that the reduction in the levels of these amino acids could play a key role in the reduced quality of post-thaw sperm. This notion is substantiated by the positive correlations detected here between sperm viability, mobility, and osmotic tolerance and these amino acids. Thus, it could be hypothesized that increasing the sperm content of these amino acids could improve the freezability of turkey semen and, consequently, the post-thaw sperm quality. This is also consistent with previous research involving both human (Renard et al.,1996) and other mammalian species (Kundu et al., 2011; Sánchez-Partida et al., 1998; Koskinen et al.,1989; Sangeeta et al., 2015; Ahme et al., 2020; Beheshti et al., 2011) in which the supplementation of amino acids was successfully used to improve post-thawing sperm quality. More recently, amino acid supplementation was successfully checked in chicken (Thananurak et al., 2020; Bernal et al., 2020; Khiabani et al., 2017; Partyka et al., 2017).

To date, the mechanism by which amino acids could provide cryoprotection is not fully understood and remains unclear (Sangeeta et al., 2015). However, some authors have proposed that amino acids could form a layer over the sperm surface via the electrostatic interaction with the phosphate group of the sperm plasma membrane phospholipids, acting as a cushion for damage against ice crystal formation and therefore preventing thermal shock (Kundu et al., 2001). Thus, amino acids could also interact with phospholipid bilayers during freezing, allowing stabi- lization of the cell membrane (Partyka and Niżański 2022).

In addition, the supplementation of amino acids to semen diluents can lead to a reduction in the concentration of toxic solutes to levels associated with lesser toxicity; moreover, some amino acids can protect sperm cells against the denaturing effects of hyperosmolality during cryopreservation process (Partyka and Niżański 2022; Heber et al., 1971). In accordance with our results, in a recent study, the supplementation of valine to chicken freezing extenders resulted in a decrease in DNA fragmentation and a positive effect on the fertilizing ability of frozen–thawed sperm, with a better response in a breed that is considered to have the lowest semen freezability (Bernal et al., 2020).

In addition to the cryoprotective role played by exogenous amino acids as extender supplementation, putative effects of endogenous amino acids in specific sperm function should be

taken in consideration. It was shown, in fact, that seminal plasma levels of Leu were higher in high- than in low-fertility bulls (Kumar et al., 2015); this was proposed to be due to the action of Leu in modulating active Ca_2^+ transport across sperm membrane, which would result in a delay in Ca_2^+ uptake in ejaculated sperm. Leu was also proposed to be one of the fertility biomarkers in bovine species (Kumar et al., 2015).

Accordingly, free Leu content in chicken seminal plasma was positively correlated with sperm viability as well as DNA integrity (Santiago-Moreno and Blesbois 2020).

Contrary to levels of the majority of detected amino acids, Gly levels increased in frozen–thawed samples, and a significant negative correlation between its levels and sperm osmotic tolerance (SOT) was found. This resembles what was already observed in a previous study, in which a decrease in SOT was associated with increased levels of Gly in sperm of ageing turkey males (Iaffaldano et al.,2018). Surprisingly enough, Gly content was also found to be negatively correlated with sperm motility. This seems to be in contrast with other reports showing that the addition of Gly to the diluent prevented significant changes in chicken sperm motility during the freezing–thawing procedure (Cerolini et al.,2007). However, this dissonant result could be due to differences in metabolism between the two avian species (Iaffaldano et al., 2013) as well as a different mechanism of action for Gly as a function of its concentration (Cerolini et al.,2007). Nonetheless, further investigation into the role of amino acids in turkey sperm metabolism should be carried out in future studies.

Other water-soluble metabolites. Similarly to what was observed for most amino acids, significant reductions in carnitine, lactate, formate, creatine, and AMP content were found in cryopreserved sperm.

Furthermore, positive correlations between the aforementioned metabolites and sperm quality parameters were found, suggesting that these metabolites could somehow be involved in the decrease in sperm quality occurring in cryopreservation.

It is not surprising that a reduced post-thawed semen quality could be related to low content of carnitine. It is known, in fact, that carnitine plays a key role in sperm metabolism by providing readily available energy, thus affecting sperm motility and maturation and the spermatogenic process (Agarwal and Said 2004). This should be more evident in turkey sperm, which presents a highly oxidative metabolism (Iaffaldano et al., 2004,2013). Moreover, carnitine has a protective action against reactive oxygen species (ROS) by exerting antioxidant properties (Vessey et al., 2021; Vicari and Calogero2001). The results therein were consistent with previous studies reporting that the addition of L- carnitine in the extender enhanced chicken sperm motility in vitro during liquid storage and frozen state (Partyka and Niżański 2022; Partyka et al., 2017; Tabatabaei and Aghaei

2012) and that supplementation of L-carnitine in the diet could improve drake semen quality (Aldaraji and Tahir 2014). Accordingly, L-carnitine supplementation to freezing extender improved human sperm motility and vitality and reduced sperm DNA oxidation during cryopreservation (Banihani et al., 2014).

The reduction in free carnitine levels observed in frozen–thawed sperm could also be due to the slight, but significant, increase in ac-carnitine levels observed in these sam- ples, which was correlated with a sperm quality decrease. Since ac-carnitine seems to be involved in buffering or trapping the excessive production of acetyl-CoA (Jeulin and Lewin 1996), it could be speculated that as a result of cryopreservation process, overproduction of acetyl-CoA could occur, which in turn could increase ac-carnitine levels in sperm. In agreement with this hypothesis, cryopreserved samples also showed an increase in citrate, which derives from the condensation between oxaloacetate and acetyl-CoA. Thus, in frozen–thawed sperm, the increases in the amounts of both ac-carnitine and citrate, together with the unvaried levels of other Krebs cycle compounds such as fumarate, may suggest a reduced capability of mitochondria to utilize acetyl-CoA through this pathway and/or a reduced capability of the mitochondrial respiratory chain (linked to the Krebs cycle by its products NADH and $FADH_2$) to support sperm energy requirement. In this regard, it must be noted that among avian species, turkey presents a high oxidative metabolism (Iaffaldano et al., 2016). The high aerobic metabolism for this species, in fact, was already reported (Wishart 1972) and further highlighted by a previous study in which it was found that the stimulation of the cytochrome c oxidase (complex IV of the respiratory chain) by He–Ne laser light increased post-thaw sperm motility in turkey, but not in chicken or pheasant (Iaffaldano et al., 2013).

In addition to mitochondrial oxidative phosphorylation, glycolysis constitutes the other energy source in sperm. In this regard, it was recently reported that water-soluble extract of quail cloacal gland secretion contained glucose as an energy source for the intrinsic sperm mobility after transportation to female vagina (Sasanami et al., 2015). However, in partial agreement with the hypothesis of a cryopreservation-dependent reduction in mitochondrial activity, here we found no differences in the levels of glucose between fresh and cryopreserved samples. Thus, in our case, a reduced sperm cell capability of glucose uptake due to putative impairment of glucose carriers should be ruled out. Contrarily, in other species such as boars, it was found that the cryopreservation process impaired glucose uptake by affecting the distribution of glucose transporters, especially GLUT-3 (Sancho et al., 2007), a GLUT family member that is also present in avians, as shown by proteomic and peptidomic analyses of chicken sperm (Labas et al.,2015). On the other hand, the impairment of the glycolytic pathway as a result of cryopreservation also seems unlikely in light

of the supposed increased acetyl-CoA production discussed above.

Despite unaltered glucose levels, in cryopreserved samples, a significant reduction in lactate content was found, which was correlated with the decreased sperm quality in these samples. This positive corelation is not surprising, since L-lactate was found to play an active role in sperm bioenergetics because of its mitochondrial metabolism (Paventi et al.,2015; Darr et al.,2016). In particular, because of the occurrence of a mitochondrial L-lactate dehydrogenase (Passarella et al., 2008, 2014; Paventi et al., 2017), mitochondria are able to actively metabolize this substrate for energy purposes (Paventi et al., 2015). For this reason, a reduction in sperm energy fuel such as L-lactate in cryopreserved sperm could be responsible for a decreased sperm quality. Therefore, further investigation of possible changes occurring in cryopreservation in cytosolic and mitochondrial L-lactate dehydrogenase (both protein levels and enzyme activities) represents a task to be addressed in future studies.

Myo-inositol levels were also investigated, since it has been reported that its addition to sperm could improve sperm motility (Condorelli et al., 2012) and mitochondrial membrane potential (Condorelli et al., 2011) (also see Vazquez-Levin and Verón 2020). However, as for glucose, no significant variation between fresh and frozen– thawed sample was found. Thus, myo-inositol should not play a role in the sperm quality decrease during turkey semen cryopreservation, at least in our experimental conditions.

Another interesting result obtained by analyzing the water-soluble fraction of frozen– thawed sperm was the decreased AMP content in these samples. This low AMP content could also partially explain the observed increase in citrate levels, since the Krebs cycle rate is extremely sensitive to AMP concentration (Martínez-Reyes and Chandel N.S. 2020). It is known, in fact, that AMP acts as a cell energy sensor via the AMP/ATP ratio; its increase is a signal of cell energy deficiency and leads to activation of the AMP-activated protein kinase (AMPK) (Hardie 2007), a mechanism well reported also in avian sperm (Nguyen et al., 2019). Thus, the observed reduction in the levels of AMP in cryopreserved samples is quite surprising, especially in light of the 40-fold decrease in ATP levels that was already found as a result of the freezing–thawing process of turkey sperm (Słowińska et al.,2018). In this regard, it must be noted that cell levels of AMP, ADP, and ATP give rise to the adenylate energy charge; moreover, these compounds are closely related to each other by the activity of the enzyme adenylate kinase (AK), which catalyzes the following equilibrium reaction: 2ADP = ATP + AMP. The activity of this enzyme was reported to allow ADP to partially support sperm motility (Vadnais et al., 2014), at least under energetic stress conditions (Xie et al., 2020). Since this scenario seems to occur in turkey sperm cryopreservation, as evidenced by the dramatic ATP decrease, it could be speculated that a reduction in AK activity could play a role in the sperm quality decrease. Thus, further investigation into this aspect (i.e., the occurrence and activity of AK in turkey sperm and its variation during the freezing–thawing procedure), as well as the detection of ADP levels in sperm before and after

cryopreservation, appears to be mandatory in further studies.

Lipids. It is widely accepted that lipids are the main component of sperm membrane and that they are responsible for the fluidity of membrane bilayers (Sanocka and Kurpisz 2004), which in turn influences the spermatozoa freezability of different animal species (Yu et al., 2022; Iaffaldano et al., 2018). Accordingly, one study showed that the freezing–thawing process resulted in a rigidifying effect on the sperm membrane and suggested that sperm adaptability to freezing–thawing-induced stress could be dependent on its initial membrane fluidity (Giraud et al., 2000). The same authors argued that the initial membrane fluidity had practical implications for predicting the response of spermatozoa following freezing and thawing and for improving the recovery of viable spermatozoa.

Thus, by turning our attention to lipid extract, in this study, we observed significant reductions in CHO, UFA, PUFA and an increase in SFA in frozen compared with fresh sperm. In addition, cholesterol was positively correlated with sperm viability, motility, and osmotic tolerance. This outcome leads us to assume that the reduction in sperm quality after freezing–thawing could be due to increased spermatozoon membrane rigidity accompanied by the decrease in the cholesterol/phospholipid ratio. This would be consistent with previous studies in human (Giraud et al., 2000). and in turkey (Blesbois et al., 2005, Partyka and Niżański 2021) reporting that semen storage and, more importantly, the freezing–thawing procedure induced a rigidifying process in the sperm membrane followed by a dramatic decrease in the cholesterol/phospholipid ratio.

Moreover, besides the cholesterol content, membrane fluidity depends on the degree of saturation of fatty acids in membrane phospholipids (Ladha 1998), since saturated fatty acids are rigidifying components of the mammalian sperm membranes. The avian sperm membrane contains more polyunsaturated fatty acids (PUFAs) than mammal sperm and has a lower protein content, a lower cholesterol/phospholipid ratio, and greater overall fluidity at physiological temperatures (Partyka and Niżański 2021). Here, we found an increase in the SFA/UFA ratio occurring in cryopreservation, since it was 0.62 and 0.75 in fresh and frozen–thawed sperm, respectively. Thus, we can conclude that, distinctly from semen liquid storage, in which SFA/UFA ratio was not affected (Douard et al., 2000), more drastic storage conditions such as cryopreservation strongly affect the lipid composition, and consequently the fluidity, of turkey sperm membrane. In this regard, previous studies showed that the lipid composition of avian spermatozoa could be modified by the diet with subsequent effects on membrane fluidity (Zaniboni et al., 2006, 2009, Bongalhardo et al., 2009). Thus, a diet inducing a lower SFA/UFA ratio in turkey sperm could be tried out in the future as a putative way to increase the freezability of avian sperm. At the same time, it must be taken in consideration that sperm cryopreservation strongly increases ROS production (Ansari et

al., 2021, Zhang et al., 2021), resulting in lipid peroxidation. Therefore, the reduction in sperm quality could also be due to a membrane destabilization deriving from PUFA peroxidation (Partyka and Niżański 2021). Thus, a higher UFA content in the sperm membrane may not necessarily be the best way to improve sperm freezability. However, proper knowledge of the relationship between metabolic profile and the freezability of the spermatozoa remains very interesting in light of the possibility of modifying the sperm metabolic profile via various factors such as diet manipulation, strain, and ageing (Zaniboni et al., 2006, 2009; Long 2006; Iaffaldano et al., 2018).

5.Conclusions

We are confident that the findings reported here provide a valid contribution to the scientific community, since they returned a clearer scenario of metabolic changes occurring in turkey sperm cryopreservation. Semen cryopreservation is an important biotechno- logical strategy used to both preserve and protect genetic resources, which are subject to increasingly serious reductions in some species, as well as to enhance animal biodiversity in the case of inbreeding risks. The knowledge of metabolites responsible for the post- thawing sperm quality decrease allows (i) identifying several biological markers related to the sperm freezability of male turkey donators and (ii) suggesting a supplementation of specific metabolites in the diet or in the freezing medium in order to obtain spermatozoa abler to withstand the freezing process.

In addition, the analytic approach used here, which resorted to the NMR technique to determine the metabolites involved in semen cryopreservation, constitutes an important tool that can be applied also to other species, including humans.

Author Contributions: Conceptualization, N.I., G.P. and M.D.I.; methodology, M.D.I., N.I., G.P., A.P.S. and L.M.; validation, S.C., L.M. and N.I.; formal analysis, N.I., M.D.I., A.P.S., L.M. and G.P.; investigation, M.S., E.A. and G.R.; data curation, G.P., M.D.I. and A.P.S.; writing—original draft preparation, G.P., N.I. and M.D.I.; writing—review and editing, G.P., N.I., S.C., L.M. and M.D.I.; visualization, G.P. and M.D.I.; supervision, N.I.; funding acquisition, N.I. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by The Italian Ministry of Agriculture, MIPAAFT-NRDP 2014- 2020-Measure 10.2 Biodiversity—Poultry sector (DG DISR-DISR07-Prot. n. 0194727-28.04.2021) project name (Conservation of biodiversity in Italian poultry breeds: insights and monitoring— TuBAvI-2).

Institutional Review Board Statement: The handling of animals and semen collection was conducted in accordance with the Code of Ethics of the EU Directive 2010/63/EU.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used are available from the corresponding author on reasonable request.

Acknowledgments: The authors thank Carmine Marini for help with semen collection and the "Amadori" group for allowing to use their breeders.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Blesbois, E.; Seigneurin, F.; Grasseau, I.; Limouzin, C.; Besnard, J.; Gourichon, D.; Coquerelle, G.; Rault, P.; Tixier-Boichard, M. Semen Cryopreservation for Ex Situ Management of Genetic Diversity in Chicken: Creation of the French Avian Cryobank. *Poult. Sci.* **2007**, *86*, 555–564. [CrossRef] [PubMed]
2. Partyka, A.; Niżański, W. Advances in storage of poultry semen. *Anim. Reprod. Sci.* **2022**, 106921. [CrossRef] [PubMed]
3. Liptoi, K.; Buda, K.; Rohn, E.; Drobnyak, A.; Meleg, E.E.; Palinkas-Bodzsar, N.; Vegi, B.; Barna, J. Improvement of the application of gonadal tissue allotransplantation in the in vitro conservation of chicken genetic lines. *Anim. Reprod. Sci.* **2020**, *213*, 106280. [CrossRef] [PubMed]
4. Santiago-Moreno, J.; Blesbois, E. Animal board invited review: Germplasm technologies for use with poultry. *Animal* **2022**, *16*, 100475. [CrossRef]
5. Blesbois, E. Freezing avian semen. *Avian Biol. Res.* **2011**, *4*, 52–58. [CrossRef]
6. Kowalczyk, A.; Łukaszewicz, E. Simple and Effective Methods of Freezing Capercaillie (*Tetrao urogallus* L.) Semen. *PLoS ONE* **2015**, *10*, e0116797. [CrossRef]
7. Thélie, A.; Bailliard, A.; Seigneurin, F.; Zerjal, T.; Tixier-Boichard, M.; Blesbois, E. Chicken semen cryopreservation and use for the restoration of rare genetic resources. *Poult. Sci.* **2019**, *98*, 447–455. [CrossRef]
8. Prentice, J.R.; Anzar, M. Cryopreservation of Mammalian Oocyte for Conservation of Animal Genetics. *Vet. Med. Int.* **2011**, *2011*,146405. [CrossRef]
9. Iaffaldano, N.; Di Iorio, M.; Rusco, G.; Antenucci, E.; Zaniboni, L.; Madeddu, M.; Marelli, S.; Schiavone, A.; Soglia, D.; Buccioni, A.; et al. Italian semen cryobank of autochthonous chicken and turkey breeds: A tool for preserving genetic biodiversity. *Ital. J. Anim. Sci.* **2021**, *20*, 2022–2033. [CrossRef]
10. Iaffaldano, N.; Di Iorio, M.; Cerolini, S.; Manchisi, A. Overview of Turkey Semen Storage: Focus on Cryopreservation—A Review.*Ann. Anim. Sci.* **2016**, *16*, 961–974. [CrossRef]
11. Blanco, J.M.; Gee, G.; Wildt, D.E.; Tselutin, K.; Donoghue, A.M. Semen cryopreservation in poultry and non-domestic species: A comparative approach to understanding the fundamentals of avian spermatozoa cryobiology. *Br. Poult. Sci.* **2000**, *41*, 3–5. [CrossRef]Blanco, J.M.; Long, J.A.; Gee, G.; Donoghue, A.M.; Wildt, D.E. Osmotic tolerance of avian spermatozoa: Influence of time, temperature, cryoprotectant and membrane ion pump function on sperm viability. *Cryobiology* **2008**, *56*, 8–14. [CrossRef] [PubMed]
12. Blesbois, E. Current status in avian semen cryopreservation. *World's Poult. Sci. J.* **2007**, *63*, 213–222.

[CrossRef]
13. Iaffaldano, N.; Romagnoli, L.; Manchisi, A.; Rosato, M.P. Cryopreservation of turkey semen by the pellet method: Effects of variables such as the extender, cryoprotectant concentration, cooling time and warming temperature on sperm quality determined through principal components analysis. *Theriogenology* **2011**, *76*, 794–801. [CrossRef]
14. Iaffaldano, N.; Di Iorio, M.; Miranda, M.; Zaniboni, L.; Manchisi, A.; Cerolini, S. Cryopreserving turkey semen in straws and nitrogen vapour using DMSO or DMA: Effects of cryoprotectant concentration, freezing rate and thawing rate on post-thaw semen quality. *Br. Poult. Sci.* **2016**, *57*, 264–270. [CrossRef]
15. Iaffaldano, N.; Rosato, M.; Manchisi, A.; Centoducati, G.; Meluzzi, A. Comparison of different extenders on the quality characteristics of turkey semen during storage. *Ital. J. Anim. Sci.* **2016**, *4*, 513–515. [CrossRef]
16. Iaffaldano, N.; Rosato, M.; Manchisi, A.; Centoducati, G.; Meluzzi, A. Comparison of different extenders on the quality characteristics of turkey semen during storage. *Ital. J. Anim. Sci.* **2016**, *4*, 513–515. [CrossRef]
17. Izanloo, H.; Soleimanzadeh, A.; Bucak, M.N.; Imani, M.; Zhandi, M. The effects of varying concentrations of glutathione and trehalose in improving microscopic and oxidative stress parameters in Turkey semen during liquid storage at 5 °C. *Cryobiology* **2021**, *101*, 12–19. [CrossRef]
18. Blesbois, E.; Grasseau, I.; Seigneurin, F. Membrane fluidity and the ability of domestic bird spermatozoa to survive cryopreserva- tion. *Reproduction* **2005**, *129*, 371–378. [CrossRef]
19. Di Iorio, M.; Rusco, G.; Iampietro, R.; Maiuro, L.; Schiavone, A.; Cerolini, S.; Iaffaldano, N. Validation of the Turkey Semen Cryopreservation by Evaluating the Effect of Two Diluents and the Inseminating Doses. *Animals* **2020**, *10*, 1329. [CrossRef]
20. Di Iorio, M.; Rusco, G.; Iampietro, R.; Colonna, M.A.; Zaniboni, L.; Cerolini, S.; Iaffaldano, N. Finding an Effective Freezing Protocol for Turkey Semen: Benefits of Ficoll as Non-Permeant Cryoprotectant and 1:4 as Dilution Rate. *Animals* **2020**, *10*, 421. [CrossRef]
21. Mosca, F.; Madeddu, M.; Abdel Sayed, A.; Zaniboni, L.; Iaffaldano, N.; Cerolini, S. Combined effect of permeant and non-permeant cryoprotectants on the quality of frozen/thawed chicken sperm. *Cryobiology* **2016**, *73*, 343–347. [CrossRef] [PubMed]
22. Zaniboni, L.; Rizzi, R.M.; Cerolini, S. Combined effect of DHA and α-tocopherol enrichment on sperm quality and fertility in the turkey. *Theriogenology* **2006**, *65*, 1813–1827. [CrossRef] [PubMed]
23. Iaffaldano, N.; Manchisi, A.; Gambacorta, M.; Di Iorio, M.; Rosato, M.P. Effect of different sperm concentrations on the post-thaw viability and motility of turkey spermatozoa cryopreserved by the pellet method. *Ital. J. Anim. Sci.* **2009**, *8*, 760–762. [CrossRef]
24. Mosca, F.; Zaniboni, L.; Abdel Sayed, A.; Madeddu, M.; Iaffaldano, N.; Cerolini, S. Effect of dimethylacetamide and N- methylacetamide on the quality and fertility of frozen/thawed chicken semen. *Poult. Sci.* **2019**, *98*, 6071–6077. [CrossRef]

25. Mosca, F.; Zaniboni, L.; Sayed, A.A.; Iaffaldano, N.; Soglia, D.; Schiavone, A.; Cerolini, S. Effect of N-Methylacetamide Concentra- tion and Thawing Rate on Chicken Sperm Quality after Cryopreservation. *Animals* **2020**, *10*, 824. [CrossRef]
26. Parks, J.E.; Lynch, D.V. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. *Cryobiology* **1992**, *29*, 255–266. [CrossRef]
27. Cerolini, S.; Zaniboni, L.; Maldjian, A.; Gliozzi, T. Effect of docosahexaenoic acid and α-tocopherol enrichment in chicken sperm on semen quality, sperm lipid composition and susceptibility to peroxidation. *Theriogenology* **2006**, *66*, 877–886. [CrossRef]
28. Blesbois, E.; Grasseau, I.; Seigneurin, F.; Mignon-Grasteau, S.; Saint Jalme, M.; Mialon-Richard, M.M. Predictors of success of semen cryopreservation in chickens. *Theriogenology* **2008**, *69*, 252–261. [CrossRef]
29. Long, J.A.; Purdy, P.H.; Zuidberg, K.; Hiemstra, S.-J.; Velleman, S.G.; Woelders, H. Cryopreservation of turkey semen: Effect of breeding line and freezing method on post-thaw sperm quality, fertilization, and hatching. *Cryobiology* **2014**, *68*, 371–378. [CrossRef]
30. Mussa, N.J.; Ratchamak, R.; Ratsiri, T.; Vongpralub, T.; Boonkum, W.; Semaming, Y.; Chankitisakul, V. Lipid profile of sperm cells in Thai native and commercial roosters and its impact on cryopreserved semen quality. *Trop. Anim. Health Prod.* **2021**, *53*, 321. [CrossRef]
31. Mandal, R.; Badyakar, D.; Chakrabarty, J. Role of Membrane Lipid Fatty Acids in Sperm Cryopreservation. *Adv. Androl.* **2014**, *2014*, 190542. [CrossRef]
32. Zaniboni, L.; Cerolini, S. Liquid storage of turkey semen: Changes in quality parameters, lipid composition and susceptibility to induced in vitro peroxidation in control, n-3 fatty acids and alpha-tocopherol rich spermatozoa. *Anim. Reprod. Sci.* **2009**, *112*, 51–65. [CrossRef] [PubMed]
33. Long, J.A. Avian semen cryopreservation: What are the biological challenges? *Poult. Sci.* **2006**, *85*, 232–236. [CrossRef] [PubMed]
34. Shinitzky, M.; Yuli, I. Lipid fluidity at the submacroscopic level: Determination by fluorescence polarization. *Chem. Phys. Lipids* **1982**, *30*, 261–282. [CrossRef]
35. Vickram, A.S.; Ramesh Pathy, M.; Sridharan, T.B. Effect of various biomolecules for normal functioning of human sperm for fertilization: A review. *Int. J. Pharm. Pharm. Sci.* **2012**, *4*, 18–24.
36. Cheah, Y.; Yang, W. Functions of essential nutrition for high quality spermatogenesis. *Adv. Biosci. Biotechnol.* **2011**, *02*, 182–197. [CrossRef]
37. Atessahin, A.; Bucak, M.N.; Tuncer, P.B.; Kızıl, M. Effects of antioxidant additives on microscopic and oxidative parameters of Angora goat semen following the freeze–thawing process. *Small Rumin. Res.* **2008**, *77*, 38–44. [CrossRef]
38. Ugur, M.R.; Dinh, T.; Hitit, M.; Kaya, A.; Topper, E.; Didion, B.; Memili, E. Amino Acids of Seminal Plasma Associated with Freezability of Bull Sperm. *Front. Cell Dev. Biol.* **2020**, *7*, 347. [CrossRef]
39. Santiago-Moreno, J.; Bernal, B.; Perez-Cerezales, S.; Castaño, C.; Toledano-Díaz, A.; Esteso, M.C.;

Gutierrez-Adan, A.; López- Sebastián, A.; Garcia-Gil, M.; Woelders, H.; et al. Seminal plasma amino acid profile in different breeds of chicken: Role of seminal plasma on sperm cryoresistance. *PLoS ONE* **2019**, *14*, e0209910. [CrossRef]

40. Renard, P.; Grizard, G.; Francoisgriveaua, J.; Sion, B.; Boucher, D.; Le Lannou, D. Improvement of Motility and Fertilization Potential of Postthaw Human Sperm Using Glutamine. *Cryobiology* **1996**, *33*, 311–319. [CrossRef]
41. Li, Y.; Si, W.; Zhang, X.; Dinnyes, A.; Ji, W. Effect of amino acids on cryopreservation of cynomolgus monkey (*macaca fascicularis*) sperm. *Am. J. Primatol.* **2003**, *59*, 159–165. [CrossRef] [PubMed]
42. Khlifaoui, M.; Battut, I.; Bruyas, J.F.; Chatagnon, G.; Trimeche, A.; Tainturier, D. Effects of glutamine on post-thaw motility of stallion spermatozoa: An approach of the mechanism of action at spermatozoa level. *Theriogenology* **2005**, *63*, 138–149. [CrossRef] [PubMed]
43. Bucak, M.N.; Tuncer, P.B.; Sarıözkan, S.; Ulutas¸, P.A. Comparison of the effects of glutamine and an amino acid solution on post-thawed ram sperm parameters, lipid peroxidation and anti-oxidant activities. *Small Rumin. Res.* **2009**, *81*, 13–17. [CrossRef]
44. Moradi, B.; Faramarzi, A.; Ghasemi-Esmailabad, S.; Aghaz, F.; Hashemian, A.H.; Khazaei, M. L-proline as a novel additive to cryopreservation media improved post-thaw quality of human spermatozoon via reducing oxidative stress. *Andrologia* **2022**, *54*, e14301. [CrossRef] [PubMed]
45. Jeulin, C.; Lewin, L.M. Role of free L-carnitine and acetyl-L-carnitine in post-gonadal maturation of mammalian spermatozoa.*Hum. Reprod. Update* **1996**, *2*, 87–102. [CrossRef]
46. Agarwal, A.; Said, T.M. Carnitines and male infertility. *Reprod. Biomed. Online* **2004**, *8*, 376–384. [CrossRef]
47. Stradaioli, G.; Sylla, L.; Zelli, R.; Chiodi, P.; Monaci, M. Effect of L-carnitine administration on the seminal characteristics of oligoasthenospermic stallions. *Theriogenology* **2004**, *62*, 761–777. [CrossRef]
48. Yu, J.; Wang, Z.; An, X.; Liu, H.; Wang, F.; Ji, C.; Liu, G.; Wang, Y.; He, F.; Dang, R.; et al. Metabolomic profiling of Dezhou donkey sperm associated with freezability. *Theriogenology* **2022**, *181*, 131–139. [CrossRef]
49. Iaffaldano, N.; Di Iorio, M.; Mannina, L.; Paventi, G.; Rosato, M.P.; Cerolini, S.; Sobolev, A.P. Age-dependent changes in metabolic profile of turkey spermatozoa as assessed by NMR analysis. *PLoS ONE* **2018**, *13*, e0194219. [CrossRef]
50. Tselutin, K.; Narubina, L.; Mavrodina, T.; Tur, B. Cryopreservation of poultry semen. *Br. Poult. Sci.* **1995**, *36*, 805–811. [CrossRef]
51. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917. [CrossRef] [PubMed]
52. Ingallina, C.; Maccelli, A.; Spano, M.; Di Matteo, G.; Di Sotto, A.; Giusti, A.M.; Vinci, G.; Di Giacomo, S.; Rapa, M.; Ciano, S.; et al. Chemico-Biological Characterization of Torpedino Di Fondi® Tomato Fruits: A Comparison with San Marzano Cultivar at Two Ripeness Stages.

Antioxidants **2020**, *9*, 1027. [CrossRef] [PubMed]

53. Ingallina, C.; Spano, M.; Sobolev, A.P.; Esposito, C.; Santarcangelo, C.; Baldi, A.; Daglia, M.; Mannina, L. Characterization of Local Products for Their Industrial Use: The Case of Italian Potato Cultivars Analyzed by Untargeted and Targeted Methodologies. *Foods* **2020**, *9*, 1216. [CrossRef] [PubMed]
54. Lemoine, M.; Mignon-Grasteau, S.; Grasseau, I.; Magistrini, M.; Blesbois, E. Ability of chicken spermatozoa to undergo acrosome reaction after liquid storage or cryopreservation. *Theriogenology* **2011**, *75*, 122–130. [CrossRef]
55. Long, J.A.; Guthrie, H.D. Validation of a rapid, large-scale assay to quantify ATP concentration in spermatozoa. *Theriogenology* **2006**, *65*, 1620–1630. [CrossRef]
56. Słowińska, M.; Liszewska, E.; Judycka, S.; Konopka, M.; Ciereszko, A. Mitochondrial membrane potential and reactive oxygen species in liquid stored and cryopreserved turkey (*Meleagris gallopavo*) spermatozoa. *Poult. Sci.* **2018**, *97*, 3709–3717. [CrossRef]
57. Kundu, C.N.; Das, K.; Majumder, G.C. Effect of Amino Acids on Goat Cauda Epididymal Sperm Cryopreservation Using a Chemically Defined Model System. *Cryobiology* **2001**, *42*, 21–27. [CrossRef]
58. Sánchez-Partida, L.G.; Setchell, B.P.; Maxwell, W.M.C. Effect of compatible solutes and diluent composition on the post-thaw motility of ram sperm. *Reprod. Fertil. Dev.* **1998**, *10*, 347–358. [CrossRef]
59. Koskinen, E.; Junnila, M.; Katila, T.; Soini, H. A Preliminary Study on the Use of Betaine as a Cryoprotective Agent in Deep Freezing of Stallion Semen. *J. Veter. Med. Ser. A* **1989**, *36*, 110–114. [CrossRef]
60. Sangeeta, S.; Arangasamy, A.; Kulkarni, S.; Selvaraju, S. Role of amino acids as additives on sperm motility, plasma membrane integrity and lipid peroxidation levels at pre-freeze and post-thawed ram semen. *Anim. Reprod. Sci.* **2015**, *161*, 82–88. [CrossRef]
61. Ahmed, H.; Jahan, S.; Khan, A.; Khan, L.; Ullah, H.; Riaz, M.; Ullah, K.; Ullah, F. Supplementation of l-tryptophan (an aromatic amino acid) in tris citric acid extender enhances post-thaw progressive motility, plasmalemma, mitochondrial membrane potential, acrosome, and DNA integrities, and in vivo fertility rate of buffalo (*Bubalus bubalis*) bull spermatozoa. *Cryobiology* **2020**, *92*, 117–123. [CrossRef] [PubMed]
62. Beheshti, R.; Asadi, A.; Eshratkhah, B.; Kandi, J.G.G.; Ghorbani, A. The effect of cysteine on post-thawed buffalo bull (*Bubalus bubalis*) sperm parameters. *Adv. Environ. Biol.* **2011**, *5*, 1260–1263.
63. Thananurak, P.; Chuaychu-Noo, N.; Thélie, A.; Phasuk, Y.; Vongpralub, T.; Blesbois, E. Different concentrations of cysteamine, ergothioneine, and serine modulate quality and fertilizing ability of cryopreserved chicken sperm. *Poult. Sci.* **2020**, *99*, 1185–1198. [CrossRef] [PubMed]
64. Bernal, B.; Iglesias-Cabeza, N.; Sánchez-Rivera, U.; Toledano-Díaz, A.; Castaño, C.; Pérez-Cerezales, S.; Gutiérrez-Adán, A.; López- Sebastián, A.; García-Casado, P.; Gil, M.; et al. Effect of

supplementation of valine to chicken extender on sperm cryoresistance and post-thaw fertilization capacity. *Poult. Sci.* **2020**, *99*, 7133–7141. [CrossRef] [PubMed]

65. Khiabani, A.B.; Moghaddam, G.; Kia, H.D. Effects of adding different levels of Glutamine to modified Beltsville extender on the survival of frozen rooster semen. *Anim. Reprod. Sci.* **2017**, *184*, 172–177. [CrossRef] [PubMed]
66. Partyka, A.; Rodak, O.; Bajzert, J.; Kochan, J.; Niżański, W. The Effect of L-Carnitine, Hypotaurine, and Taurine Supplementation on the Quality of Cryopreserved Chicken Semen. *BioMed Res. Int.* **2017**, *2017*, 7279341. [CrossRef]
67. Heber, U.; Tyankova, L.; Santarius, K.A. Stabilization and inactivation of biological membranes during freezing in the presence of amino acids. *Biochim. Biophys. Acta Biomembr.* **1971**, *241*, 578–592. [CrossRef]
68. Kumar, A.; Kroetsch, T.; Blondin, P.; Anzar, M. Fertility-associated metabolites in bull seminal plasma and blood serum: 1H nuclear magnetic resonance analysis. *Mol. Reprod. Dev.* **2015**, *82*, 123–131. [CrossRef]
69. Santiago-Moreno, J.; Blesbois, E. Functional Aspects of Seminal Plasma in Bird Reproduction. *Int. J. Mol. Sci.* **2020**, *21*, 5664. [CrossRef]
70. Cerolini, S.; Zaniboni, L.; Mangiagalli, M.G.; Gliozzi, T.M. Effect of glycine on cryopreservation of chicken spermatozoa. *Avian Poult. Biol. Rev.* **2007**, *18*, 65.
71. Iaffaldano, N.; Paventi, G.; Pizzuto, R.; Passarella, S.; Cerolini, S.; Zaniboni, L.; Marzoni, M.; Castillo, A.; Rosato, M.P. The post-thaw irradiation of avian spermatozoa with He-Ne laser differently affects chicken, pheasant and turkey sperm quality. *Anim. Reprod. Sci.* **2013**, *142*, 168–172. [CrossRef] [PubMed]
72. Iaffaldano, N.; Meluzzi, A.; Manchisi, A.; Passarella, S. Improvement of stored turkey semen quality as a result of He–Ne laser irradiation. *Anim. Reprod. Sci.* **2005**, *85*, 317–325. [CrossRef] [PubMed]
73. Vessey, W.; Saifi, S.; Sharma, A.; McDonald, C.; Almeida, P.; Figueiredo, M.; Minhas, S.; Virmani, A.; Dhillo, W.S.; Ramsay, J.W.; et al. Baseline levels of seminal reactive oxygen species predict improvements in sperm function following antioxidant therapy in men with infertility. *Clin. Endocrinol.* **2021**, *94*, 102–110. [CrossRef] [PubMed]
74. Vicari, E.S.D.; Calogero, A.E. Effects of treatment with carnitines in infertile patients with prostato-vesiculo-epididymitis. *Hum.Reprod.* **2001**, *16*, 2338–2342. [CrossRef] [PubMed]
75. Tabatabaei, S.; Aghaei, A. Effect of l-carnitine on sperm quality during liquid storage of chicken semen. *Comp. Clin. Pathol.* **2012**,*21*, 711–717. [CrossRef]
76. Aldaraji, H.J.; Tahir, A.O. Effect of L-carnitine supplementation on drake semen quality. *S. Afr. J. Anim. Sci.* **2014**, *44*, 18. [CrossRef]
77. Banihani, S.; Agarwal, A.; Sharma, R.; Bayachou, M. Cryoprotective effect of l-carnitine on motility, vitality and DNA oxidation of human spermatozoa. *Andrologia* **2014**, *46*, 637–641.

[CrossRef]

78. Wishart, G.J. Maintenance of ATP concentrations in and of fertilizing ability of fowl and turkey spermatozoa in vitro. *Reproduction* **1982**, *66*, 457–462. [CrossRef]
79. Sasanami, T.; Izumi, S.; Sakurai, N.; Hirata, T.; Mizushima, S.; Matsuzaki, M.; Hiyama, G.; Yorinaga, E.; Yoshimura, T.; Ukena, K.; et al. A unique mechanism of successful fertilization in a domestic bird. *Sci. Rep.* **2015**, *5*, 7700. [CrossRef]
80. Sancho, S.; Casas, I.; Ekwall, H.; Saravia, F.; Rodriguez-Martinez, H.; Rodriguez-Gil, J.E.; Flores, E.; Pinart, E.; Briz, M.D.; Garcia-Gil, N.; et al. Effects of cryopreservation on semen quality and the expression of sperm membrane hexose transporters in the spermatozoa of Iberian pigs. *Reproduction* **2007**, *134*, 111–121. [CrossRef]
81. Labas, V.; Grasseau, I.; Cahier, K.; Gargaros, A.; Harichaux, G.; Teixeira-Gomes, A.-P.; Alves, S.; Bourin, M.; Gérard, N.; Blesbois, E. Qualitative and quantitative peptidomic and proteomic approaches to phenotyping chicken semen. *J. Proteom.* **2015**, *112*, 313–335. [CrossRef]
82. Paventi, G.; Lessard, C.; Bailey, J.L.; Passarella, S. In boar sperm capacitation l -lactate and succinate, but not pyruvate and citrate, contribute to the mitochondrial membrane potential increase as monitored via safranine O fluorescence. *Biochem. Biophys. Res. Commun.* **2015**, *462*, 257–262. [CrossRef] [PubMed]
83. Darr, C.R.; Varner, D.D.; Teague, S.; Cortopassi, G.A.; Datta, S.; Meyers, S.A. Lactate and Pyruvate Are Major Sources of Energy for Stallion Sperm with Dose Effects on Mitochondrial Function, Motility, and ROS Production. *Biol. Reprod.* **2016**, *95*, 34. [CrossRef] [PubMed]
84. Passarella, S.; de Bari, L.; Valenti, D.; Pizzuto, R.; Paventi, G.; Atlante, A. Mitochondria and L-lactate metabolism. *FEBS Lett.* **2008**, *582*, 3569–3576. [CrossRef] [PubMed]
85. Paventi, G.; Pizzuto, R.; Passarella, S. The occurrence of l-lactate dehydrogenase in the inner mitochondrial compartment of pig liver. *Biochem. Biophys. Res. Commun.* **2017**, *489*, 255–261. [CrossRef]
86. Passarella, S.; Paventi, G.; Pizzuto, R. The mitochondrial L-lactate dehydrogenase affair. *Front. Neurosci.* **2014**, *8*, 407. [CrossRef]
87. Condorelli, R.A.; La Vignera, S.; Bellanca, S.; Vicari, E.; Calogero, A.E. Myoinositol: Does it improve sperm mitochondrial function and sperm motility? *Urology* **2012**, *79*, 1290–1295. [CrossRef]
88. Condorelli, R.A.; La Vignera, S.; Di Bari, F.; Unfer, V.; Calogero, A.E. Effects of myoinositol on sperm mitochondrial function in-vitro. *Eur. Rev. Med. Pharmacol. Sci.* **2011**, *15*, 129–134.
89. Vazquez-Levin, M.H.; Verón, G.L. Myo-inositol in health and disease: Its impact on semen parameters and male fertility. *Andrology* **2020**, *8*, 277–298. [CrossRef]
90. Martínez-Reyes, I.; Chandel, N.S. Mitochondrial TCA cycle metabolites control physiology and disease. *Nat. Commun.* **2020**, *11*, 102. [CrossRef]
91. Hardie, D.G. AMP-activated/SNF1 protein kinases: Conserved guardians of cellular energy. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 774–785. [CrossRef]

92. Nguyen, T.M.D.; Grasseau, I.; Blesbois, E. New insights in the AMPK regulation in chicken spermatozoa: Role of direct AMPK activator and relationship between AMPK and PKA pathways. *Theriogenology* **2019**, *140*, 1–7. [CrossRef] [PubMed]
93. Vadnais, M.L.; Cao, W.; Aghajanian, H.K.; Haig-Ladewig, L.; Lin, A.M.; AlAlao, O.; Gerton, G.L. Adenine Nucleotide Metabolism and a Role for AMP in Modulating Flagellar Waveforms in Mouse Sperm1. *Biol. Reprod.* **2014**, *90*, 128. [CrossRef] [PubMed]
94. Xie, M.; Zhang, G.; Zhang, H.; Chen, F.; Chen, Y.; Zhuang, Y.; Huang, Z.; Zou, F.; Liu, M.; An, G.; et al. Adenylate kinase 1 deficiency disrupts mouse sperm motility under conditions of energy stress. *Biol. Reprod.* **2020**, *103*, 1121–1131. [CrossRef]
95. Sanocka, D.; Kurpisz, M. Reactive oxygen species and sperm cells. *Reprod. Biol. Endocrinol.* **2004**, *2*, 12. [CrossRef] [PubMed]
96. Shabani, S.; Mehri, M.; Shirmohammad, F.; Sharafi, M. Enhancement of sperm quality and fertility-related parameters in Hubbard grandparent rooster fed diets supplemented with soybean lecithin and vitamin E. *Poult. Sci.* **2022**, *101*, 101635. [CrossRef] [PubMed]
97. Giraud, M.N.; Motta, C.; Boucher, D.; Grizard, G. Membrane fluidity predicts the outcome of cryopreservation of human spermatozoa. *Hum. Reprod.* **2000**, *15*, 2160–2164. [CrossRef] [PubMed]
98. Partyka, A.; Niżański, W. Supplementation of avian semen extenders with antioxidants to improve semen quality—Is it an effective strategy? *Antioxidants* **2021**, *10*, 1927. [CrossRef]
99. Ladha, S. Lipid heterogeneity and membrane fluidity in a highly polarized cell, the mammalian spermatozoon. *J. Membr. Biol.* **1998**, *165*, 1–10. [CrossRef]
100. Douard, V.; Hermier, D.; Blesbois, E. Changes in Turkey Semen Lipids During Liquid In Vitro Storage1. *Biol. Reprod.* **2000**, *63*, 1450–1456. [CrossRef]
101. Bongalhardo, D.C.; Leeson, S.; Buhr, M.M. Dietary lipids differentially affect membranes from different areas of rooster sperm. *Poult. Sci.* **2009**, *88*, 1060–1069. [CrossRef] [PubMed]
102. Ansari, M.S.; Rakha, B.A.; Akhter, S.; Akhter, A.; Blesbois, E.; Santiago-Moreno, J. Effect of glutathione on pre and post-freezing sperm quality of Indian red jungle fowl (*Gallus gallus murghi*). *Theriogenology* **2021**, *172*, 73–79. [CrossRef] [PubMed]
103. Zhang, B.; Wang, Y.; Wu, C.; Qiu, S.; Chen, X.; Cai, B.; Xie, H. Freeze-thawing impairs the motility, plasma membrane integrity and mitochondria function of boar spermatozoa through generating excessive ROS. *BMC Vet. Res.* **2021**, *17*, 127. [CrossRef] [PubMed]

CONCLUSIONS AND FUTURE PERSPECTIVES

The current doctoral thesis constitutes a notable contribution to the conservation and restoration of genetic biodiversity in aquatic and avian species with a specific focus on investigating the impact of ovarian fluid on fertilization success in native Mediterranean trout. The research activities of my doctoral thesis were conducted within two funded projects (TuBAvI-2 and LIFE Nat.Sal.Mo) with a shared objective of preserving and enhancing the targeted species. This entailed maintaining genetic resources within a germplasm cryobank and improving artificial reproduction techniques to increase genetic variability.

In the context of Mediterranean trout, the utilization of cryobanks to support the conservation of endangered native species has the potential to alleviate or resolve the known issues affecting supportive breeding and broodstock management. This contributes significantly to preserving wild genetic biodiversity and preventing domestication. To further advance the adoption of these practices, the establishment of operational cryopreservation and fertilization protocols is paramount. In this regard, our study proposes that the presence of ovarian fluid, either alone or in conjunction with D-532, within the artificial microenvironment of reproduction is a crucial factor in enhancing the success of fertilization when utilizing frozen semen from Mediterranean brown trout. However, it is important to note that OF 100% serves as a natural and viable medium. Maintaining ovarian fluid within a reproductive microenvironment provides the advantage of shielding the eggs from potentially harmful external factors and streamlining field operations conducted along riverbanks, particularly in relation to the removal process when D-532 is used alone.

This research opens up new avenues for future exploration. For instance, it suggests the characterization of ovarian fluid biochemistry from native Mediterranean trout to delve into the components that could influence the *in vitro* and *in vivo* performance of both fresh and frozen semen. Furthermore, there's a potential to standardize fertilization protocols in controlled reproduction settings. Additionally, this research could serve as a catalyst for further investigations into the use of pure or mixed ovarian fluid as a fertilization medium in other species of aquaculture interest.

Also, intriguing findings emerged in other study that compared native ovarian fluid to spring water. Specifically, native males exhibited enhanced sperm swimming performance in the presence of native ovarian fluid, whereas ovarian fluid from non-native trout (zootechnical origin) yielded no such effect. This suggests the potential evolution of an ovarian fluid-driven recognition mechanism aimed at augmenting fertilization success in native populations of Mediterranean brown trout.

Our results indicate that native *S. cettii* females exhibit a superior ability to enhance the sperm motility of native Mediterranean trout males compared to zootechnical *S. trutta*. This suggests a potential finely-tuned mechanism favoring egg-sperm encounters. However, to conclusively confirm these fertilization outcomes, it would be imperative to conduct *in vivo* fertilization tests, replicating the

critical natural conditions such as flowing water, turbulences, and dynamics of gamete expulsion. These findings have significant implications for genetic biodiversity conservation, contributing to protecting species threatened by genetic introgression with domestic strains.

Lastly, two additional studies were undertaken to conserve the genetic resources of chicken and turkey breeds, emphasizing the importance of cryobanks and delving into the molecular mechanisms underlying sperm cryopreservation to enhance the process. The activities outlined in the paper *"Italian semen cryobank of autochthonous chicken and turkey breeds: a tool for preserving genetic biodiversity*" are instrumental in establishing the Italian avian semen cryobank and serve as general guidelines applicable to similar initiatives in birds or other domestic animals. The establishment of the first Italian semen cryobank for native chicken and turkey breeds represents a valuable tool for safeguarding and conserving biodiversity within Italian poultry populations still present in farming systems. The creation of an accessible cryobank can provide breeders with semen doses from males of high genetic value while simultaneously aiding in the mitigation of inbreeding within farms.

The integration of emerging technologies, including genomics, molecular biology, and NMR, holds the potential to significantly enhance our understanding of conservation and preservation practices concerning animal genetic resources. These advancements enable a deeper comprehension of genetic diversity and evolutionary connections both within and among species.

In our research "*The Effect of Semen Cryopreservation Process on Metabolomic Profiles of Turkey Sperm as Assessed by NMR Analysis*", we utilized NMR techniques among emerging technologies as a valuable tool to enhance comprehension of the biological foundations of turkey semen cryopreservation. We identified biological markers associated with the sperm freezability of male turkey donors, suggesting a potential supplementation of specific metabolites in either the diet or the freezing medium to bolster the resilience of spermatozoa during the freezing process. This discovery offers a significant contribution to the scientific community given the pivotal role of semen cryopreservation in preserving and safeguarding genetic resources, particularly amid the escalating threat of decreasing populations in certain breeds/species and the imperative to counteract inbreeding risks to enhance animal biodiversity. Moreover, the analytical methodology employed herein, exploiting NMR techniques to delineate the metabolites implicated in semen cryopreservation, represents a crucial tool that holds applicability across various species, including humans.

Our research aligns perfectly with the objectives of the National Regional Strategic Plan (PSRN) as it contributes to the preservation of the Italian avian genetic heritage, promoting biodiversity and innovation in the livestock sector. Furthermore, it aims to enhance native breeds in sustainable farming systems, agritourism, and social agriculture.

In conclusion, the future of genetic biodiversity conservation depends on continued commitment to research and the implementation of innovative practices. By leveraging acquired knowledge and adopting a multidisciplinary approach, we can hope to ensure the survival and prosperity of threatened species/breeds for future generations.

LIST OF PUBLICATIONS

Publications in International Journals with I.F.

- Nicolaia Iaffaldano, Michele Di Iorio, Giusy Rusco, **Emanuele Antenucci**, Luisa Zaniboni, Manuela Madeddu, Stefano Marelli, Achille Schiavone, Dominga Soglia, Arianna Buccioni, Martino Cassandro, Cesare Castellini, Margherita Marzoni, Silvia Cerolini. *Italian semen cryobank of autochthonous chicken and turkey breeds: a tool for preserving genetic biodiversity*. Italian Journal of Animal Science; (2021) https://doi.org/10.1080/1828051X.2021.1993094
- Paventi G, Di Iorio M, Rusco G, Sobolev AP, Cerolini S, **Antenucci E**, Spano M, Mannina L, Iaffaldano N. *The Effect of Semen Cryopreservation Process on Metabolomic Profiles of Turkey Sperm as Assessed by NMR Analysis.* Biology. (2022); 11(5):642. https://doi.org/10.3390/biology11050642
- Rusco G., Di Iorio M., Esposito S., **Antenucci E**., Roncarti A., Iaffaldano N. (2023). *The Use of Ovarian Fluid as Natural Fertilization Medium for Cryopreserved Semen in Mediterranean Brown Trout: The Effects on Sperm Swimming Performance.*VETERINARY SCIENCES, 10, 219. https://doi.org/10.3390/vetsci10030219
- Rusco G., Di Iorio M., Esposito S., Gibertoni P.P., **Antenucci E.,** Palombo V., Roncarti A., Iaffaldano N. (2023). *Zootechnical brown trout (Salmo trutta L. 1758) ovarian fluid fails to upregulate the swimming performances of native Mediterranean brown trout (Salmo cettii Rafinesque, 1810) sperm in the Biferno River*. FISHES, 8, 190. https://doi.org/10.3390/fishes8040190
- Di Iorio, M., Lauriola, F., Rusco, G., **Antenucci, E.,** Schiavitto, M., & Iaffaldano, N. (2024). *Cryopreserving Rabbit Semen: Impact of Varying Sperm Concentrations on Quality and the Standardization of Protocol*. Veterinary sciences, 11(1), 9. https://doi.org/10.3390/vetsci11010009

Publications at Conference/Congress

- *La crioconservazione del seme come strategia per la salvaguardia della biodiversità: la prima criobanca in Europa per la conservazione della Trota mediterranea nativa dei fiumi molisani (Life NAT.SAL.MO Project – Regione Molise)"* Rusco G., Di Iorio M., **Antenucci E.,** Esposito S., Iaffaldano N. XIII XIII National Congress on Biodiversity (Biodiversity 2021) Foggia 7-9 September 2021.

- *Tutela delle razze cunicole italiane mediante l'implementazione della prima criobanca del seme a livello nazionale."* Di Iorio M., Rusco G., **Antenucci E**., Schiavitto M., Marsia M., Iaffaldano N. XIII National Congress on Biodiversity (Biodiversity 2021). Foggia 7-9 September 2021.
- *"Cryobanking of native Mediterranean brown trout semen as a tool for the safeguard of genetic resources."* Iaffaldano N, Rusco G., Esposito S., **Antenucci E**., Di Iorio M. 1. 24th Congress ASPA - Padova (Italy) 21-24 September 2021.
- *"Implementation of the first Italian semen cryobank of autochthonous turkey breeds: preliminary results".* Iaffaldano N., Di Iorio M., Rusco G., **Antenucci E**., Zaniboni L., Cerolini S. 24th Congress ASPA - Padova (Italy) 21-24 September 2021.
- *"The effectiveness of ovarian fluid on spermatozoa performances in Mediterranean brown trout wild population (Molise region - Italy)".* Giusy Rusco, Stefano Esposito, Michele Di Iorio, **Emanuele Antenucci**, Alessandra Roncarati, Nicolaia Iaffaldano. 8th International Workshop on the Biology of Fish Gametes. Gdansk (Polonia).
- *"Phenotypic characterization of semen production and quality in Italian chicken and turkey breeds."* Di Iorio M., Iaffaldano N., Rusco G., **Antenucci E**., Madeddu M., Zaniboni L., Marelli S., Cerolini S. (2023) XXV National Congress ASPA Monopoli (Bari - Italy), 13-16 June 2023, ITALIAN JOURNAL OF ANIMAL SCIENCE, 22 (1): 101, abstract O090.
- *"The supportive breeding of native Mediterranean brown trout (LIFE Nat.Sal.Mo project, Molise region): the effect of ovarian fluid on frozen sperm motility parameters during artificial fertilization."* Rusco G., Di Iorio M., Esposito S., **Antenucci E**., Lerza L., Roncarati A., Iaffaldano N. (2023). XXV National Congress ASPA Monopoli (Bari - Italy), 13-16 June 2023, ITALIAN JOURNAL OF ANIMAL SCIENCE, 22 (1): 105, abstract O048.
- *"The first semen cryobank in Europe for the protection of Mediterranean brown trout wild populations: the results over four years within the LIFE Nat.Sal.Mo project (Molise region - South Italy)."* Iaffaldano N., Rusco G., Esposito S., Roncarati A., **Antenucci E**., Lerza L., Di Iorio M. (2023). XXV National Congress ASPA Monopoli (Bari - Italy), 13-16 June 2023, ITALIAN JOURNAL OF ANIMAL SCIENCE, 22 (1): 225, abstract P088.
- *"Italian semen cryobank of autochthonous chicken breeds: the case study of Siciliana breed".* Di Iorio M., Rusco G., **Antenucci E.,** Lerza L., Zaniboni L., Madeddu M., Iaffaldano N. *(2023).* XXV National Congress ASPA Monopoli (Bari - Italy), 13-16 June 2023, ITALIAN JOURNAL OF ANIMAL SCIENCE, 22 (1): 226, abstract P089.

- *"Carcass and meat quality traits of Siciliana chickens, a native Italian breed".* Tavaniello S., Iaffaldano N., Peng M., Grassi G., Di Iorio M., **Antenucci E**., Palazzo M., MAIORANO G. (2023). EAAP Regional Meeting 2023, 26-28 April 2023, Nitra (Slovakia).
- *"Uso de fuentes ricas en Ácidos Grasos de Cadena Media en alimentaciòn de gallinas ponedoras y su efecto sobre el rendimiento y la calidad del huevo."* Orozco, C. Garcès-Narrom J.A: Garcia-Bautista, M. Palomar, **E.Antenucci**, R. Sala, L. Guiot, M.D. Soler. LVII Poultry Symposium Avila 2023.
- *"Exploring Phenotypic Traits and Immunological Features in the Native Sicilian Chicken Breed."* **Emanuele Antenucci,** Michele Di Iorio ,Giusy Rusco, Nicolaia Iaffaldano. UNIMOL PHD EXPO 2nd edition.

Oral Communication

- The 8th "Aquatic Biodiversity" International Conference, Sibiu, Romania, European Union, September 20-22, 2022. **Emanuele Antenucci**, Oral Presentation titled: "The effect of ovarian fluid on the motility of cryopreserved sperm of native Mediterranean trout *(S. macrostigma*)."