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Animal Genetics
Approaches and Limitations

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ANIMAL GENETICS - APPROACHES AND LIMITATIONS

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Meet the editor

Dana Liana Pusta is currently a Professor of Animal Genetics, Molecular Genetics, and Hereditary Diseases in Domestic Animals at the Faculty of Veterinary Medicine, USAMV Cluj-Napoca, Romania. She received her BSc in 1996 (veterinary medicine - FVM Cluj-Napoca), and her PhD title with "cum laude" distinction in 2005. She has published three editions of genetic textbooks for students (two in Romanian and one in English) and more than 150 research papers. She is an EAEVE (European Association of Establishments for Veterinary Education) evaluator in the basic sciences field and she is a member of ECA (European Cytogenetics Association) and EBTNA (European Biotechnology Thematic Network Association) associations. She is co-author of two patents and 3 trademarks. She was awarded with different distinctions, such as: Diploma and Medal for the Progress of Veterinary Medicine, Best Biotechnological Product Award, and Medal of Honor of FVM Cluj-Napoca.

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Introductory Chapter: Challenges and Advances in Animal Genetics

Dana Liana Pusta

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1. Introduction
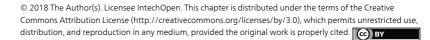
Genetics is a science which studies the mechanisms and biological laws that provide stability of the features during generations, meaning heredity on the one hand and the variation of inherited characteristics on the other hand [1, 2].

Genetics studies the three main fundamental properties of the living organisms such as heredity, variability, and reproduction [1].

The term heredity comes from Greek (*gr. Hereditas*), which means to inherit. Biologically, heredity refers to the inheritance of some "features and characters" from the parents to the descendants. In fact, not the characters themselves are inherited, but the potentiality of their apparition, intermediated by some material particles named genes (*gr. genan = to produce, to give birth*). The resulted gametes do not possess the parental characters, but they possess the parental genes which are transmitted to the future organism. The resulted zygote will form a new organism which will have particular features and characters, which will only resemble the parents to a certain extent.

Basically, the characters concerning the species are inherited, but not the features which are specific to each individual. This phenomenon represents the basis of variability of the living creatures. Reproduction assures the substrate of heredity and variability of the living world, determining at the same time the evolution of life.

Genetic processes are fundamental in order to understand life itself. Genetic information is stored in the DNA molecules since the formation of the first cell—the zygote, which controls all cellular functions, determines the external appearance of all organisms, and serves as a link between generations in all species. So, it is obvious that understanding of genetics is essential to fully understand life in all its aspects [3].

Whether we like it or not, heredity is a phenomenon linked to the existence of living matter, and it is one of the fundamental expressions of life, manifesting itself all over nature as long as life will exist [3].

2. The main branches of animal genetics

Classically, animal genetics is considered to be divided into a few important branches, such as:

Basic genetics—which enframes **Cytogenetics** and **Mendelian genetics**. **Cytogenetics** basically studies the chromosomes and the cell life (cell cycle, mitosis, and meiosis), and **Mendelian genetics** refers to the application of the Mendelian laws and their exceptions in animal inheritance.

Molecular genetics—studies the molecular basis of the heredity such as nucleic acids, proteins (biological active molecules), and the relations between them.

Genetic engineering—is defined as an ensemble of methods and technologies made with genes, chromosomes or even entire cells, on the purpose of obtaining new genetic structures with deliberate hereditary features.

Heredopathology (medical genetics)—refers to the study of illnesses, syndromes, and genetic abnormalities. Another important branch related with Heredopathology is **genetic prophylaxy**, which tries to elucidate the etiological factors which determine a genetic disorder and its organism-environment relation. **Immunogenetics** is also considered as a branch of medical genetics that explores the relationship between the immune system and genetics.

Population genetics—studies the inheritance pattern of different features which are manifested and transmitted in different populations of animals.

In recent years, new concepts are developed, based on what generally is named "systems biology" which represents the biological research which is mainly focused on the systematic study of complex interactions in biological systems by using integration models [4]. This concept of system biology is directly linked to "Omic sciences and omic technologies" and to the general concept of "systems genetics" which combine genetics and system biology [5].

The omic sciences and technologies are considered to be consisted of some new concepts which are linked together, such as **genomics, genome; transcriptomics and transcriptome; proteomics and proteome; metabolomics, metabolome and metabolomics** and omic experiments.

Genomics studies the structure, function, and expression of all genes in an organism [4]. The term **genome** was introduced by German botanist Hans Winkler and represents the total number of the chromosomes in ova or spermatozoa in humans or animals [6], or in other words, the genome means the total DNA of a cell or organism [4] or the total genes of an organism. The **transcriptomic** studies the formation, maturation, and role of mRNA in a cell or in an organism, and the **transcriptome** is the total mRNA which results in a cell or an organism

[4]. As an analogy with the term genomics, another term is defined as **proteomics** meaning the large-scale study of proteins regarding their formation, structure, and function in a cell or an entire organism as a system. The **proteome** represents the set of all expressed proteins in a cell, tissue, or organism [4].

Metabolomics studies the global metabolite profile in a biological system under a given set of conditions [4]. The **metabolome** is represented by the total quantitative collection of low molecular weight compounds (metabolites) which are formed and are present in a cell or organism that are directly involved in metabolic reactions. It also includes those metabolites taken in from external environment or provided by symbiotic relationships [4]. **Metabonomics** represents the measure of the fingerprint of biochemical perturbations caused by diseases, drugs, and toxins (**Figure 1**) [4].

Beside what happens in the interior of a cell or of an organism, the external factors from the environment must also be taken into considerations when we discuss about the exteriorization of a genotype. This is mainly the reason why a new term was introduced into the genetical studies such as **epigenetics**. The term "epigenetics" was introduced to the lexicon in the year 1942 by the developmental biologist and evolutionist Conrad H. Waddington by combining the word "genetics" with "epigenesis" in order to describe the influence of the environmental cues on the development of specific phenotypes due to the genotype-environment interactions [7, 8]. In 2008 at Cold Spring Harbor meeting [9], the epigenetic trait was defined as "a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" also considered as synonym for "epigenetic inheritance" (**Figure 2**) [8].
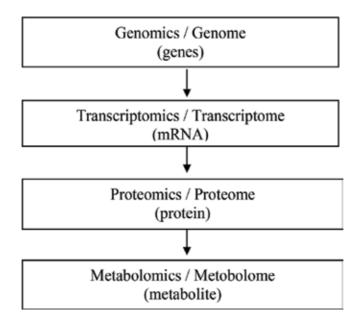
Figure 1. Omic sciences and their interactions. The flow of biological information is bidirectional [4].
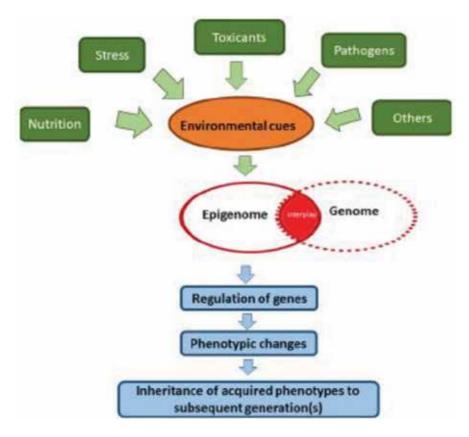
Figure 2. Graphical abstract of epigenetical inheritance [8].

Behavioral genetics is also a new part of genetics of the twenty-first century which studies "whether and how much genetic factors influence behavior to ask questions about development, about relations among traits, and about the interplay between nature and nurture. The identification of specific genes associated with behavior will make it possible for behavioral scientists to ask more precise questions about how genotypes become phenotypes" [10].

3. The role of animal genetics

Animal genetics, at present, is considered as a science with important theoretical and practical significance in order to find answers for the actual problems of mankind, related to the need for food, food with improved features, improvement of animal populations, and also for solving different issues related to human and animal health.

Genetics field supports the understanding of the process of inheritance during generations, the changing of genetic structures of different animal populations, and also the quantitative and qualitative improvement of animal productions [3].

The morphological and functional inheritance features are generally considered as being specific to each species, on the condition that the animal development is made under the same environmental conditions. In reality, in the environment, there are permanent changes and challenges, which will lead to different particular phenotypical manifestations in the frame of the individuals belonging to the same species, breed, or even line [3].

The inheritance pattern is given by the trinity of molecules: DNA, RNA, and proteins [8]. The DNA represents the genetic material in mammals, which is organized into genes which store the genetic information since the formation of the zygote. The genetic information stored into different parts of DNA is first transferred to a closely related mRNA and then into the cytoplasm of the cell, where the ribosomes are. There, the information stored in the RNA is translated into amino acids, which will be linked together to form the proteins with structural and functional role. The diverse functions of proteins determine the biochemical identity of cells and also determine the expression of inherited traits [8].

So, life is based on the process of storage and expression of genetic information, which is encoded in the individual DNA since the beginning of its life and it is permanently influenced by the environment [3].

Omic technology can be applied on one hand to better understand the physiological, normal processes in the entire body system, and, on the other hand to screen, diagnose, prognose, and understand the etiology of a disease [4]. The omic investigation is also used in drug discovery and assessment of their toxicity and efficacity, especially in cancer and cardiac therapies [4].

The discovery of new techniques and technologies generally related to omic sciences opens new perspectives and new discoveries in the study of the molecular mechanisms of the organisms, but there are also some limitations, both regarding the techniques themselves and the interpretation of the data.

4. Conclusions

1. Animal genetics as a science is a relatively new field of biology studies.

2. Classically, some fields of animal genetic studies are described, such as basic genetics (cytogenetics and Mendelian genetics); molecular genetics, genetic engineering; medical genetics (heredopathology); and population genetics.

3. During the last decades, new fields of animal genetics were developed, mainly due to the integrated concept of system genetics, considering the cell, tissue, and sometimes the entire organism as an integrated system.

4. System genetics is directly linked to omic sciences and omic technologies, which comprise the complex and bidirectional relations between genomics and genome; transcriptomics and transcriptome; proteomics and proteome; metabolomics and metabolome; and metabolomics and omic experiments.

5. The interactions on the genotype produced by the environment are studied by behavioral genetics and epigenetics in a complex inter-relation resulting in the changing in the phenotype.

6. The new discoveries in animal genetics are opening new perspectives regarding animal breeding, control, prevention, and treatment of animal diseases.

7. The new techniques and technologies have some limitations regarding the technologies themselves and also regarding the interpretation of the results.

8. In conclusion, we can say that the new genetic approaches are solving some important problems, but also at the same time, science must face the new problems which are arisen by the application and interpretation of the new genetic technologies.

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Relationship between Plasma Proteins and Boar Semen Freezability

Francisco Javier Henao

Additional information is available at the end of the chapter

Abstract

Currently, there is great interest in using frozen boar semen to enhance pig-breeding processes. Yet semen freezability, as well as its limited lifespan in the uterus, limits the efficacy of such a procedure. Pig spermatozoa membrane is less stable and more sensitive to low temperatures as it contains lower levels of cholesterol. It is also highly susceptible to lipid peroxidation (LPO) during freezing, since it is rich in polyunsaturated fatty acids (PUFA). Seminal plasma (SP) has beneficial effects on post-thaw semen quality and its composition may have a genetic basis, specifically in protein content. To date, studies on boar semen freezability have focused on sperm cell proteins with very little attention having been paid to SP proteins. In boar SP, there are 82 identified proteins with spermadhesins (90%) and fibronectins (FN) the most abundant. The only plasma protein thus far identified as a freezability marker is FN1. Other plasmatic proteins of recognized importance in the freezing of porcine semen are: DQH, HSP90AA1, NPC2, L-PGDS, ß-HEX, SOD, and PON-1. The purpose of this chapter is to examine the most efficacious elements of the above plasma proteins with regard to their role as biological or potential biological markers of porcine semen freezability.

Keywords: pigs, reproduction, boar, semen freezability, plasma proteins

1. Introduction

Despite the excellent results in fertility and prolificacy achieved with fresh-cooled boar semen in commercial pork production operations [1], interest in the use of frozen boar semen persists. Applications of frozen boar semen include: the transfer of genes between population pyramids; availability of in-farm insemination plans; the export of germplasm; prevention of particular pathogenic transmission agents; and the establishment of germplasm banks [2].

However, there are clear constraints on the use of frozen semen in commercial pig breeding. Most salient among these are the extremely high volumes and concentrations required per artificial insemination (AI) dose, as well as lower farrowing rates and litter size [1, 3–5]. Such issues are mainly caused by the variability in the capacity of boar spermatozoa to survive the freezing and thawing process (freezability) and the shortened lifespan of frozen–thawed boar sperm in the uterus [6–8]. Despite following the best-known cryopreservation protocols, seminal quality is affected to such an extent [3], that quality may vary as much as 70% [9]. Genetic factors [8] and proteomics variations [10–13] are the most commonly cited reasons for such variances.

Farrowing rates using frozen semen now approach the 85–90% success rate already seen in semen refrigeration techniques, thanks to innovative deep intrauterine insemination and sow management methods [9, 14]. Insemination using frozen–thawed boar sperm should not be performed using conventional AI but rather through post-cervical or deep intrauterine insemination [15–18].

Lower levels of cholesterol exhibited within its cell membrane renders pig sperm more sensitive to cold and less stable [1]. This is evidenced by a molar ratio cholesterol to phospholipids of 0.26 (the bull has a ratio of 0.45) [19]. This makes the sperm more prone to initiate the capacitation and acrosome reaction process [20, 21]. The freezing of porcine semen reduces the sperm survival rate by more than 50%, while causing most of the surviving cells to prematurely develop a phenomenon similar to capacitation—cryo-capacitation [22, 23]. Sperm capacitation is a biochemical process that the sperm must undergo during the passage through the reproductive tract of the female on their way to fertilizing an oocyte [24, 25].

Sperm membranes are rich in polyunsaturated fatty acids (PUFA), and therefore highly susceptible to lipid peroxidation (LPO) in conditions of oxidative stress by the increase of free oxygen radicals or reactive oxygen species (ROS), [26, 27]. Within these cells, the oxidative stress can be induced by different endogenous and exogenous factors that are activated during their passage through the male and female reproductive tracts [28]. As a counterbalance, there are multiple protection mechanisms that reduce oxidative injury [29].

It is also known that seminal plasma (SP) has beneficial effects on post-thaw semen quality [30]. SP is a mixture of secretions from the testicular network, the epididymis, and the accessory sex glands [25, 31] and there is evidence that its composition may have a genetic basis [8] and varies among boars [32, 33], specifically in its protein content [25, 30, 32]. All these suggest that SP is a factor closely related to freezability. SP of the pre-sperm fraction originates in the urethral and bulbourethral glands [34, 35]; SP of the fraction rich in sperm comes from the prostate, epididymis, and seminal vesicles; and SP of the sperm-poor fraction originates solely from the prostate and seminal vesicles [25]. SP is the sole vehicle in which sperm is immersed after ejaculation [25, 31] and regulates processes related to nutrition, protection, maturation, motility, and sperm capacitation [30, 36, 37].

Findings show that differences in freezability between males disappear when, (1) cryopreserving sperm obtained directly from the epididymis (which has had no contact with SP) [38] and, (2) are minimized when freezing the sperm peak fraction poor in SP and abundant in sperm (the first 10 ml of the rich fraction) [30]. Moreover, after mixing high and

low freezability SP and sperm from boars, the combination of high freezability sperm and plasma from boars recorded the highest value of acrosome integrity, while the addition of SP from high freezability boars to any category of sperm cell, yielded the highest values of membrane structural integrity [39].

The effect of SP on sperm function is extremely variable, and although several proteins and their effects have been identified, little is known about their wider effects and potential applications. It is therefore necessary to examine these molecules and how they interact and impact on cell function [40]. SP is composed of water, inorganic ions, citric acid, organic salts, prostaglandins, and proteins. The latter has been linked to having the greatest effect on sperm function and freezability [32]. The literature touches on SP's cryoprotective effect on capacitation and survival of spermatozoa [31], together with its positive correlation to membrane structural integrity, acrosome integrity, sperm motility, and mitochondrial membrane potential after manipulation of semen [35, 39].

The purpose of the following chapter is to review the most relevant aspects of plasma proteins recognized as biological or potential biological markers of porcine semen freezability.

2. Plasmatic proteins and your relationship with semen freezability

Current studies on the relationship between protein markers of boar semen freezability have been focused on sperm cell proteins with very little attention being paid to seminal plasma proteins [12]. Tandem mass spectrometry of two-dimensional liquid chromatography (2D–LC) derived samples identifying a total of 82 proteins in the seminal plasma of the boar [41]. Proteins with the greatest presence in the porcine SP are spermadhesins (90%) and fibronectins (FN) [32, 41]. Other plasmatic proteins of recognized importance in the freezing of porcine semen that are unconfirmed as freezability markers are: DQH protein [42, 43], Heat shock protein 90 alpha A1 (HSP90AA1), Niemann-pick disease type C2 protein (NPC2), Prostaglandin D synthase lipocalin type (L-PGDS) [44], ß subunit of N-acetyl-b-hexosaminidase (ß-HEX) [45], superoxide dismutase (SOD), and paraoxonase 1 (PON-1) [46]. The only plasma protein identified as a freezability marker is Fibronectin 1 (FN1) [12].

2.1. Spermadhesins

The spermadhesins are a family of sperm surface-associated glycoproteins of 12–16 kDa, possessing between 109 and 133 amino acids, and constituted by a single-domain CUB that serves as a structural support [32]. The spermadhesinas are multifunctional proteins that have the capacity to unite different ligands. These include: heparin, phospholipids, protease inhibitors, and carbohydrates. These change with glucosylation and aggregation, and can be divided into heparin-binding (AQN-1, AQN-2, AQN-3, AWN-1 and AWN-2) and non-heparin-binding (PSP-I and PSP-II) families [34, 47–50]. AWN, AQN-1, AQN-3, PSP-I, and PSP-II spermadhesins are transcribed and translated in seminal vesicles and the prostate. mRNA transcripts of these spermadhesins have been detected in caudal epididymis, and, in the case of PSP-I, have found mRNA in caput epididymis and rete testis [51]. The spermadhesins increase as the

sperm concentration decreases, resulting in a greater concentration of spermadhesins in the sperm-poor fraction [25, 52]. The PSP-I and PSP-II spermadhesins make up 50% of the SP proteins [47]. These bind to the sperm membrane in the acrosomal domain [53], and, in the case of PSP-II, are known to bind to phosphorylcholine [42], forming a cover that protects sperm from premature capacitation [32, 47]. When PSP spermadhesins are added to the semen in sperm sexing and freezing processes, they reduce the percentage of sperm with high concentrations of intracellular calcium, and maintain the survival, motility, and integrity of the mitochondrial membrane [47, 52]. In addition, it is known that exposure of frozen–thawed semen to low concentrations of PSP-I and PSP-II spermadhesins maintains viability and sperm motility but also has an inhibitory effect on the oocyte penetration rate [35]. In addition, it has been found that spermadhesins induce the migration of polymorphonuclear neutrophils within the uterine tract of the sow [54].

The spermadhesin AWN-1 makes up between 7 and 8% of the total protein of SP [55], binding to cholesterol and phosphorylcholine of the plasma membrane, during storage in the epididymis and during ejaculation [42]. This protein is considered a decapacitating factor, with 90% being released during the sperm capacitation [55]. In addition, the AWN-1 participates in the union between gametes through its ability to bind to ß-galactosides from the zona pellucida [32].

The spermadhesin AQN-1 is related to the union of the spermatozoon with the epithelium of the uterus in the reservoir located in the uterotubal junction [56]. This is due to its ability to bind to different ligands, including mannose [32]. This protein is united to the acrosome membrane, and 50–75% are released during the sperm capacitation, suggesting a decapacitating effect [55].

2.2. Fibronectins

Fibronectin (FN) is an abundant soluble constituent of plasma (300 μg/ml) and other body fluids and also part of the insoluble extracellular matrix; FN can be subdivided into two forms: soluble plasma FN (pFN) and less-soluble cellular (cFN) FN [57]. The fibronectins are products of expression of a single gene, the final protein may vary since alternative splicing of a single pre m-RNA is generated up to 20 variants in humans [58]. FN generally is a dimer with 2 similar ~250 kDa subunits linked covalently; each monomer is made up of 3 types of repeating units (type I, type II and type III) and approximately 90% of the FN sequence consists of 12 type I repeats, two type II repeats, and 15–17 type III repeats [57, 59]. All three types of FN repeat are also found in other molecules, suggesting that FN evolved through exon shuffling [59].

In boar seminal plasma, FN1 has also been identified and described as one of the most abundant proteins in the seminal plasma of this species [41, 60]. FN1 is considered a molecular marker for boar sperm freezability since it presents different concentrations of high and low freezability among males [12]. These differences were also confirmed by Rungruangsak et al. [61]. FN1 is related to defects of the intermediate tract and tail of the spermatozoon. Binding to the plasma membrane through integrins, and providing a protective protein of sperm; FN1 interacts with albumin, which reduces oxidative stress [60]. The binding of FN2 to the phosphorylcholine of the sperm membrane may prevent the movement of phospholipids and maintain membrane stability. Meanwhile, its binding to heparin may also enable cholesterol release during sperm capacitation [32].

2.3. Aspartic acid-glutamine-histidine protein or DQH protein

While DQH is a protein with a structure distinct from that of the spermadhesins, it forms a complex with spermadhesins AWN and AQN to perform similar functions [60]. This protein is produced in seminal vesicles and is not detected in spermatozoa of the epididymis [43]. It interacts with cholesterol, and also binds to phosphorylcholine of the spermatic membrane, to glycoproteins of the zona pellucida of the oocyte [42] and to epithelial cells of the oviduct. It is thus related to the formation of the spermatic reservoir and the sperm-oocyte junction [43].

2.4. Heat shock protein 90 alpha A1

In a study of the genes that code for heat shock proteins of 90 kDa (HSP90) or HSP90 (Heat shock protein 90 kDa), carried out by Chen et al. [62], 17 different genes were grouped into the following 4 classes: HSP90AA, HSP90AB, HSP90B, and TRAP. HSP90A indicates that the protein is cytosolic, HSP90B that the protein is from the reticulum endoplasmic and TRAP that the protein is mitochondrial. In addition, the HSP90A class was divided into two categories: (1) alpha (HSP90AA) to indicate that the gene is inducible and (2) beta (HSP90AB) to indicate that the gene is constitutive.

HSP90 proteins are highly conserved molecular companions that recognize hydrophobic regions exposed in denatured proteins and, in case of folding defects, to correct them in order to avoid irreversible protein aggregation [63]. These proteins intervene as protectors in oxidative stress, mediate cell repair, and increase resistance in case of persistent damage [64]; they have also been associated with cellular protection in thermal stress caused by high and low temperatures [63]. Another HSP90 function is the interruption of the apoptosis process via the interaction with and structural conservation of protein kinase B [65]. The thermal shock protein 90 alpha (inducible) A1 (cytosolic) (HSP90AA1) has been found in the sperm flagellum and has been associated with freezability in boars [13]; there are also reports of the existence of mRNA for this protein in sperm cells [66], which is synthesized from spermatogenesis and spermiogenesis [67]. The HSP90 interact with other proteins in different biochemical pathways to ensure a correct folding and proper functioning [65]. In the sperm capacitation process, the transduction signals that initiate phosphorylation are mediated by the cyclic AMP pathway and protein kinase A [24]. The phosphorylation of HSP90AA1 demonstrates the possibility of mediating this protein through threonine/serine kinases or by tyrosine kinase [68]. In addition, this protein is responsible for the signals of the activation of phosphorylation in tyrosine residues of flagellar proteins [69].

There is evidence of decreased intracellular concentration of HSP90AA1 during freezing, concomitant with reduced sperm motility, which is possibly associated with the protective function of this protein and its relationship with the activation of the enzyme nitric oxide synthase [70] and the beneficial role of nitric oxide in mobility [71]. In a study conducted by Hou et al. [72], it was found that the antibiotic geldanamycin, which is a specific inhibitor of HSP90, increases the production of nitric oxide and promotes sperm capacitation in the boar—a fact that indicates a different role of the HSP90 in these processes involving the porcine species.

HSP90AA1 is considered a molecular marker for boar semen freezability and it is found intracellularly in lower quantities in low freezability than in higher freezability spermatozoa [13].

This may be due to their exit from the spermatozoa into the extracellular space because of the loss in the integrity of the plasma membrane during the cooling [70]. The concentration of the HSP90AA1 protein increased in seminal plasma during the period from ejaculation up to 3 h of holding time, and this increase was greater among low freezability spermatozoa [44].

2.5. Niemann-pick disease type C2 protein

Niemann-pick C disease is a hereditary disease in which there is accumulation of cholesterol in the endosomes and lysosomes of the cell; it is caused by the mutation of two genes: NPC2 and NPC1 [73]. The mutation of the NPC2 gene causes a defect in the exit of cholesterol from the lysosomes, due to the absence of the Niemann-pick protein type C2 (NPC2) (also called HE1, which is expressed in lysosomes) [74]. The NPC2 protein is in different tissues and is most widely secreted in the epididymis of humans [75] and porcine [76]; it is also secreted in seminal vesicles, prostate, and vas deferens, and is therefore found in seminal plasma [77]. In the porcine species 2 isoforms of 19 and 16 kDa have been found, with differences in weight due to the greater amount of carbohydrates added by N-glycosylation in the 19 kDa. Both exhibit a micromolar affinity to cholesterol to the extent that for each mole of protein, one mole of cholesterol is attached [76]. The importance of the NPC2 protein is that it binds to the cholesterol of the sperm membrane with great efficiency [77], In contrast, NPC2 isoform 16 kDa does not have this capacity [76].

The exit of cholesterol from the membrane is a trigger of the sperm capacitation process [78] and the procedures performed on semen during freezing encourage this output. This leads to premature capacitation or cryo-capacitation [79]. NPC2 maintains the proportion of cholesterol in the sperm membrane and is a decapacitating factor [80]. In addition, this protein has a heparin-binding capacity, which suggests an action in sperm capacitation, due to the fact that heparin has a capacitating effect in bovine species [81].

Two isoforms of 16 and 19 kDa of the NPC2 protein have been found [44, 76]. The concentration of 19 kDa was higher in boars with seminal plasma of good freezability, with this concentration reducing in the period between ejaculation and the conclusion of 3 hours of interaction with spermatozoa. These results may be associated with the ability of NPC2 to bind to the cholesterol of the sperm membrane and suggest a better preventative mechanism against cryocapacitation [44]. These findings serve as a basis to confirm this protein as a new marker of boar freezability.

2.6. Prostaglandin D synthase lipocalin type

Prostaglandin D synthase type lipocalin (L-PGDS) is the only lipocalin that has enzymatic activity of prostaglandin H2 transformation, produced from arachidonic acid by cycloxygenase, in prostaglandin D2 [82]. L-PGDS is the most abundant protein in cerebrospinal fluid and is 75% similar to a homologous protein of 26 kDa. The latter is found in seminal plasma and is related to fertility in bulls [83] and boars [84]. The synthesis of this protein is mainly carried out in epididymal epithelial cells, with mRNA having also been found in Leydig cells in the testis and in prostate epithelial cells [85]. The main function of lipocalins is the transportation

of lipophilic substances, and in the case of L-PGDS, a high affinity has been recorded between it and retinoids, such as retinoic acid and retinol [86]. Stillwell and Wassall [87] reported that the presence of these two molecules in the plasma membrane affects their permeability by interacting with phospholipids, which results in greater ion entry from the outside. This could be related to capacitation, given that the beginning of the molecular cascade of this event is signaled by the change of membrane permeability to ions such as calcium, which results in acrosome reaction and hypermotility [88]. Gerena et al. [83] detected that L-PGDS is present in the acrosomal membrane in ejaculated spermatozoa, and then disappears in spermatozoa with acrosome reaction. In addition, in vitro incubation of spermatozoa with the L-PGDS protein increases the union of these with the zona pellucida [89], an action that is only possible after membrane changes that occur during capacitation [90]. L-PGDS also has an affinity for docosahexaenoic acid, of which it is also a transporter [91]. Docosahexaenoic acid interacts with other membrane lipids such as cholesterol and can play an important role in local structure and membrane function [92].

Another of the most important functions of the L-PGDS is its intervention in spermatogenesis and sperm maturation, through the thyroid hormone (triiodothyronine, T3) that regulates the growth, maturation, and functioning of Sertoli cells. This is made possible due to the ability of this protein to pass through membranes and to establish a connection between the blood fluid, the testicle, and the epididymis [83]. This protein is a potential marker of boar freezability because it has been found in different concentrations in the seminal plasma of semen with both high and low freezability [44].

2.7. ß subunit of N-acetyl-b-hexosaminidase

Among the most important enzymes related to the freezability of swine semen is the ß subunit of N-acetyl-b-hexosaminidase (ß-HEX). ß-HEX binds to the acrosome and is released during the acrosome reaction. A reliable indicator of sperm cryotolerance, this enzyme was found to be negatively correlated with motility, plasma membrane integrity, and post-thawing lipid peroxidation [45].

2.8. Antioxidants enzymes

In sperm cells, there are multiple protection mechanisms against oxidative stress [29], most saliently the antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [93]. Spermatozoa may also depend upon the presence of extracellular free-radical scavenging systems, which interact with biochemical components of the seminal plasma [94].

2.8.1. Paraoxonase type 1

The paraoxonase (PON) enzyme family is functionally linked to cholesterol and is composed of three members: PON-1, PON-2, and PON-3 [95]. PON-1 and PON-3 are extracellular enzymes present in blood plasma, while PON-2 is an intracellular enzyme not found in blood plasma [95, 96].

PON-1 is an high-density lipoprotein associated enzyme that possess antioxidant and anti-inflammatory properties, which prevents low-density lipoprotein and high-density lipoprotein oxidation and, consequently, protects cells against oxidative stress [96].

Barranco et al. [97] revealed and characterized the presence of the antioxidant enzymes PON-1 and PON-2 in boar semen. PON-1 binds to membrane cholesterol, preventing its oxidation and thereby positively influencing both motility and the sperm membrane integrity [46]. Likewise, the presence of this protein is correlated with quality, functionality of liquid-stored semen samples and can also be related to fertility outcomes in boars. Its antioxidant properties, specifically the decrease of intracellular ROS generation, could contribute to the superior ability of the spermatozoa present in the sperm-peak portion of the ejaculate and to colonize the sperm reservoir in the oviduct [98]. The sperm-peak portion contains SP with better antioxidant capacities, greater cryotolerance, and lower ROS generation than the post sperm-rich fraction. This coincides with high SOD and PON-1 values in this portion [46].

Given the fact that PON-1 is positively related to total motility and viability of frozen–thawed boar semen, [46], that there is evidence that activity levels in SP differ among boars [98], and the possibility exists that PON-1 levels are genetically determined [96], it can be concluded that PON-1 is a potential molecular marker of boar semen freezability.

2.8.2. Superoxide dismutase

Superoxide dismutases are a family of metalloenzymes involved in intracellular and extracellular antioxidant defense system by catalyzing the dismutation of superoxide anions into hydrogen peroxide and oxygen. In mammals, three SOD isoenzymes have been described by their cellular localization, metal composition in the active site, and sensitivity to inhibitors [99, 100]. It is known that there are two subtypes of copper and zinc containing SOD (Cu/Zn-SOD): cytosolic and secretory extracellular SOD, occurring in the seminal plasma (EC-SOD). However, there are no research on its properties and functions. The EC-SOD is an important antioxidant enzyme of boar seminal plasma, which plays an important physiological role in counteracting oxidative stress in spermatozoa [94].

Conflict of interest

The author declares that he does not have any competing interest.

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Behavioral Factors Affecting Reproduction in Domestic Horses: Sociobiological Approach

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Additional information is available at the end of the chapter

Abstract

Fetal loss is a common phenomenon in domestic horses, being usually highest (up to 40%) of all domestic ungulates. However, in all studies investigating this problem, sociobiological approach, such as conflict between the evolutionary interests of individuals of the two sexes, has been neglected. Here we summarize results of three consecutive studies considering infanticide as a male's reproductive strategy and a mare's counter strategies against it. When a mare was either artificially inseminated or removed from her home environment and transported elsewhere for mating and then returned back into her home environment, containing a stallion (and/or gelding) who did not sire her fetus, she got into a potentially infanticidal danger. She more likely disrupted her pregnancy than a mare living in an environment with the sire of her fetus. This was highly affected by the social environment. Lowest percentage of pregnancy disruption can be achieved when the pregnant mare was released into an enclosure shared with the non-sire male whom she can "convince" he is the father of her expected foal by promiscuous mating. The effects of social environment leading to pregnancy block and/or disruption may thus explain substantial part of the high incidence of domestic horse fetal loss.

Keywords: pregnancy disruption, domestic horse, Bruce effect

1. Introduction

Fetal loss is a common phenomenon in domestic horses, being usually substantially higher than that in other domestic ungulates such as cattle (3.62–6.9%) [1, 2], goats (13%) and sheep (7.5%) [3]. Among Sable Island feral horses, fetal loss, as deduced from fecal oestrogens, reached up to 37.3% [4]. Even in young healthy domestic horse mares, approximately 10%

of detected pregnancies were lost during the first 5 weeks of gestation, while, in older mares, many embryos died before the first pregnancy check [5].

There have been a number of studies which have sought to explain an unusually high incidence of fetal loss in domestic horses reaching up to 40% [4–14]. However, these studies, as well as many breeders and veterinarians, neglect other factors associated with reproductive fitness and female reproductive strategies across species [e. g., [15]] One of those neglected factors may be sexual conflict, a conflict between the evolutionary interests of individuals of the two sexes [16], broadly investigated in behavioral ecology and sociobiology [15]. This conflict, originally driven by natural selection, might be further altered by physical and social environment in breeding practise.

2. Infanticide

While there are miscellaneous ways of sexual conflict one of the forms of interest in context of early fetal loss is infanticide (i.e. killing of conspecific's offspring by the individuals of the same species) by males, which causes serious costs on female reproductive success [17]. According to the original sexual selection hypothesis infanticide improves male mating success by accelerating the return of females to fertilizable condition [18, 19]. In seasonal breeders the benefits of infanticide by males is in improvement of the female breeding condition [18, 20–22]. The study of red deer (*Cervus elaphus*) was the first to provide empirical support for this hypothesis [22], which has been invoked for other mammals [18]. Many authors reviewed the possible female counterstrategies to infanticide ranging from polyandrous mating [17, 18, 23–25], through pregnancy block [26–29], later called "Bruce effect" [30–33], up to individual behavior such as maternal aggression, association with coalitionary defenders of either sex, etc. [18].

2.1. Infanticide in equids

The incidence and forms of infanticide varies among numerous mammalian groups. Sociality and social organization are among the most important predictors determining infanticide [34, 35]. The variability of incidence of male infanticide among various species and even subspecies was also reported specifically in equids [36, 37]. Based on interspecific differences in social organization, male infanticide has been suggested advantageous in all equid species, such as plains zebra, *Equus burchelli*, with exception of asses, *E. africanus*, *E. hemionus*, and *E. kiang* or Grévy's zebra, *E. grevyi* [37, 38].

In line with this prediction, the male infanticides was reported in Przewalski horse, *Equus przewalskii* [39–43], mountain zebra, *Equus zebra* [44, 45], plains zebra *Equus quagga* [36, 45], and domestic horse *Equus ferus caballus* [46–48]. Equids thus represent the group of ungulates with highest reported incidence of male infanticide.

In a herd of Przewalski's horses Feh and Munkhtuya [43] investigated male's infanticide and found that infanticide did not reduce birth intervals which they interpreted as contradictory to sexual selection hypothesis. Instead they concluded that male's infanticide is either linked

to crowding or to human disturbance. Nevertheless, they did not consider any other potential benefits of premature termination of lactation by male's infanticide such as improving the female condition and thus her future reproductive success as suggested by others earlier [18, 20–22] and which has been proposed also for equids [39, 49]. Horse mares commonly become pregnant within a month or less after parturition of the previous foal [e. g., [50–52]]. They are thus sensitive to intensive maternal investment due to concurrent pregnancy and lactation. Pregnant mares cope with parallel investment into a nursed foal and a fetus through enhancing nursing behavior in early stages of pregnancy before the initially low requirements of the fetus increase [53]. Nevertheless, lowering mare's investment for costly nursing allocated to non-filial offspring by killing it may significantly advance resources invested in stallion's own progeny.

Hoesli et al. [39] suggested an additional alternative benefit of killing a dependent foal for the new-coming stallion. This might be the elevated testosterone level in a newly established harem stallion and the reluctance of mares to accept a new breeding partner. Altogether, male's infanticide in equids should be thus taken into consideration.

Our investigation to the present chapter has not been straight-forwarded. In context of those days knowledge, initially we investigated the possibility of forced copulation as an alternative to infanticide in the plains zebra. At that time, feral domestic horse mares were found to foal in alternate years or in two out of three years [54]. Killing a lactating offspring could improve the dam's condition increasing her chance to conceive within the same season. This resembled similar situation and reasoning for existence of infanticide as a male's reproductive strategy in a seasonal breeder, in red deer [22]. Foeticide as an extension to infanticide has also been reported as a candidate for improving reproductive success of a male in feral horses [55] as well as for captive plains zebra [36, 45]. Generally speaking invading males could induce abortions in females as a result of forced copulations [55] or just sexual harassment [56] and these aborting females would be then inseminated by the new males. Though, abortion subsequent to forced copulation has not been reported in numerous other feral populations of domestic horse [54, 57]. Although still interpreted as a possible forced copulation, our data on captive zebras did not show either any convincing evidence that the forced copulation by a new-coming stallion was the actual cause of abortion. What we did found, however, was that the probability of foal death was greatest when the new male joined the herd just after conception and decreased with increasing time between conception and date of the new male introduction - the chance of a foal surviving was less than 5% just after conception and more than 50% at the time of delivery [45]. The loss of pregnancy in the zebra mares after a new non-sire male joined the herd [36, 45] made us to testing the presumption that the "Bruce effect" could explain fetal loss in domestic horses [48]. It is presumed that the logic of the "Bruce effect" as a female counter-strategy to infanticide is to prevent the waste of energy in producing offspring likely to be lost [17, 18, 23, 58].

It is a common practice that the domestic horse mare is removed from her home environment and transported elsewhere for mating. After conceiving she is returned back into her home environment and social group, containing often familiar stallions and geldings [48]. If we presume that the behavioral adaptation for infanticide relevant for wild or free-ranging horses has not be lost in domestication, and it is obvious that it remained at least within feral

populations [46, 47], then we may expect that, unless prevented by fencing or other management measures, the dominant males in the home social group may subsequently attempt infanticide.

3. Reproduction failure in domestic horse mares

We have investigated the problem in three subsequent reports where we described detailed methods and statistical analysis [48, 59, 60] and hence we will avoid presenting these details here. Basic tested hypothesis was that if the domestic horse mare was removed from her home environment and transported elsewhere for mating and then returned back into her home environment, containing a stallion (and/or gelding) who is not sire of her fetus, she would be more likely to disrupt her pregnancy than a mare living in an environment with the sire of her fetus. After conceiving she is returned back into her home environment and social group, containing often familiar stallions and geldings. This basic hypothesis was tested in the first study [48]. In the second step we investigated if a mare may consider pregnancy caused by artificial insemination as a pregnancy induced by a strange stallion [59], and finally we elucidated what happens when an out of home mated mare returns into male-free environment [60].

The reproductive data analyzed in the three steps originated from a questionnaire (**Table 1**) on reproduction of individual mares distributed subsequently to private horse owners in the Czech Republic. As the conditions under which the mares could contact (at least visual or vocal) home males differed among facilities, we used the term "enclosure" for any fenced area in which the horses were kept, regardless of the presence or absence of grass.

3.1. Reproduction failure after mating mares out of home and bringing them back to an environment containing a stallion and/or gelding

We obtained data for mares kept with one or more stallions and/or geldings present, either in the same or adjacent enclosure or further separated from the mares. All mares involved were positive in pregnancy testing after the mating and/or artificial insemination and before transportation home, which is a common routine in the country. The tests are usually performed between 2 weeks and 120 days after presumed conception [48].

In total 81 records of 60 different mares aged from 4 to 24 years, giving birth to 0 to 5 foals, and bred between years 1994 and 2008 were collected. The mares belonged to 21 breeds and came from 30 individual breeders. In 20% of cases artificial insemination was used. The age and previously successful pregnancies of the mares were (mean ± s. e. m.) $13.00 ± 0.92$ years and $1.94 ± 0.32$ foals for the mares mated with a home stallion and $12.46 ± 0.72$ years and $1.99 ± 0.25$ foals for those mated with a foreign stallion [48].

Of those mares mated with a foreign stallion outside the home stable, 31% aborted. In contrast, none of the 36 mares who conceived with a home stallion disrupted pregnancy (**Figure 1**). Five of the mares experienced both situations in different pregnancies, to be mated with a home stallion (n = 6, one mare was mated in two different seasons) and with a foreign stallion (n = 5).

Characteristic
Information on the mare:
Year of her birth
Breed *
Number of foals she had delivered
If she was nursing a foal when pregnant (Yes/No)
If she was transported during her pregnancy (Yes/No)
If she was transported during her pregnancy, then how many times
Number of failing pregnancies before the analyzed one
Type of use (Breeding/Racing/Sport/Recreational riding/Draught/Other)
Housing (Stalls/Loose boxes/Group housed in a barn/Group housed in paddocks/Pasture)
How was the mare kept on a pasture and/or in a paddock (Alone/In a group/Not at all)
Time (hours) spent daily on a pasture and/or in a paddock
Year of mating/insemination
Month of mating/insemination
How many times mated or inseminated
Transported elsewhere for mating/insemination (Yes/No)
Method of breeding (Pasture or paddock mating/In-hand breeding/Artificial insemination)
Immobilization of the mare during mating (None/On a leading rope/Lifted forelimb/Hindlimb hobbles/Twitch/Other)
Time spent with a stallion in free-running mating (days)
Date of parturition
Number of horses kept within the facility regardless of the housing system
Number of adult mares kept within the facility regardless of the housing system
Number of adult home stallions kept within the facility regardless of the housing system
Number of adult home geldings kept within the facility regardless of the housing system
Where were the adult mares kept within the facility* (Same enclosure as the pregnant mare/ out of the enclosure)
Where were the adult home stallions kept (Same enclosure as the pregnant mare/ out of the enclosure) **
Where were the adult home geldings kept (Same enclosure as the pregnant mare/ out of the enclosure) **
Conditions under which the mare was kept elsewhere if transported for mating out of her home stall:
How many days did the mare spent out of her home stall
If the mare was tested for pregnancy before returning home (Yes/No)
Number of foreign adult stallions kept within the facility where the mare was staying for mating and/or insemination
Number of foreign adult geldings kept within the facility where the mare was staying for mating and/or insemination
Where were the adult mares kept (Same enclosure as the pregnant mare/ out of the enclosure) **

Characteristic
Where were the foreign adult stallions kept (Same enclosure as the pregnant mare/ out of the enclosure) **
Where were the foreign adult geldings kept (Same enclosure as the pregnant mare/ out of the enclosure) **

*Akhal-Teke, American quarter horse, Arabian, Austrian warm-blooded horse, Belgian draft horse, Czech saddle ponies, Czech warm-blooded horse, Friesian, Furioso, Haflinger, Hanoverian, Hutsul horse, Lipizzaner, Old Kladruby horse, Purebred Spanish horse, Saxon, Silesian cold-blooded horse, Thoroughbred, Welsh cob, Welsh pony, cross-breeds.

**Detailed description where the other animals were kept was available. This included in an adjacent box, in a distant box, within the same enclosure as the mare or in the adjacent enclosure. Since this did not appear significant, the details are omitted in the table.

Table 1. Questionnaire on reproduction of the mares involved and conditions under which the mares were living during the analyzed pregnancy.

Also, in this case, none of the mares conceiving with home stallion disrupted pregnancy. On the other hand, 3 of the 5 mares (60%) conceiving with a foreign stallion aborted. Over one half (53.33%) of the mares who conceived with a home stallion were transported during their pregnancy, most of them repeatedly. None of the transported mares mated with home stallion aborted [48].

We fitted the statistical model for the mares that were transported to another facility for mating with a foreign stallion, and then returned to their home stable containing home stallion or gelding (PROC GENMOD, SAS version 9.1, designed for repeated measurements). The probability of pregnancy disruption depended on two effects only: whether the mare subsequently shared the home enclosure with at least one stallion and/or gelding (**Figure 1**) and on the number of foals the mare had delivered in the past (**Figure 2**). Pregnancy disruption was much more likely when the mare had no male company in her enclosure but one or more home stallions or geldings in adjacent enclosure were present, than when stallions and/or geldings were sharing the same enclosure with the mare (**Figure 1**, odds ratio = 6.99). With an increase in the number of foals delivered the probability for the mare to abort decreased (**Figure 2**).

Some respondents to our questionnaire reported increased sexual activity by a home stallion or dominant gelding shortly after the mare returned from mating with a foreign stallion if released into the enclosure with them. In 78.13% of cases mating of the pregnant mare by home stallion and/or gelding sharing the same enclosure was reported.

We have thus shown that in domestic horses, bringing a pregnant mare into proximity with a male who was not the father of her fetus increases probability of pregnancy disruption. In contrast to earlier studies on equids [36, 45, 55] and in agreement with Kirkpatrick and Turner [57], we suggested that forced copulations were unlikely to cause fetal loss in this study, since more losses were reported where home males were in adjoining enclosures than when they were in the same enclosure as the pregnant female. Pregnancy disruption was 7 times more likely when the mare had no male company in her enclosure but one or more home stallions or geldings were in an adjacent enclosure [48].

As reviewed by Becker and Hurst [31], "pregnancy disruption in rodents is triggered by semio-chemicals in urine, most likely oestrogens [61, 62], that are pumped into the vomeronasal organ
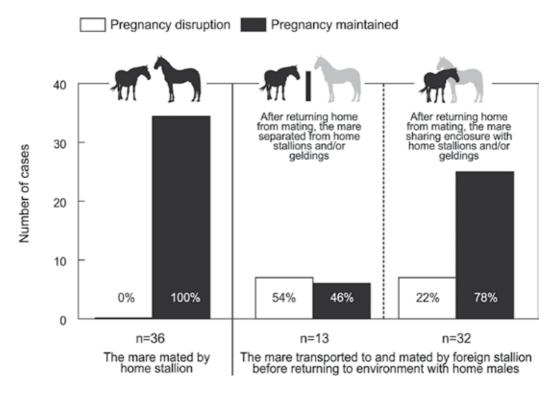
Figure 1. Proportion of pregnancy disruption in mares mated either by a home stallion or transported to and mated by a foreign stallion before they returned to environment containing home males (stallions and/or geldings) which were either separated from the returned mare or shared enclosure with her (reprinted from [48], permission for reuse provided by Springer nature).

following nasal contact with male scent. This activates a specific vomeronasal neuroendocrine pathway that inhibits prolactin release causing luteolysis and hence pregnancy failure. It was unlikely for the mares to contact with urine of the males located on the other side of the fence and to avoid possible effect of urine of the males within the same enclosure. Therefore, we can only speculate there is some other mechanism than the classical Bruce effect leading to luteolysis and hence pregnancy block in the mare" [48].

Pregnant mares in our study have been seen to be sexually active at times when conception was impossible in line with many other studies [63–68]. Such a sexual behavior can be interpreted as to confuse paternity [24, 30]. The males that had copulated with a given female are generally inhibited from killing young for the time period in which their offspring would be vulnerable to infanticide [17, 69]. Multi-male mating by females confuses paternity, thereby deterring infanticide by males. Such multiple mating is relatively common in mammals, occurring in over 100 species [25]. In many of these species, females also engage in nonprocreative mating [70]. Pregnant mares in our study isolated from home males by a fence were seen standing close to the neighboring male behind the fence, frequently urinating and soliciting him [48]. The same type of behavior was reported in rodents, primates, carnivores, and insectivores as a counter-strategy against male's infanticide [25].
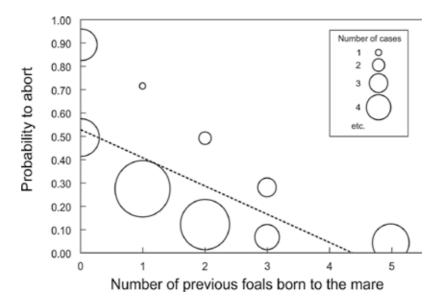
Figure 2. Predicted probability to abort by domestic mares plotted against the number of foals delivered in the past (reprinted from [48], permission for reuse provided by Springer nature).

We concluded that increased abortion among mares who had no direct access to home males (different enclosures) was as a direct result of a lack of opportunity in such situations to confuse home males about parentage. Termination of pregnancy in this way may thus be seen as an adaptation to save energy and avoid likely future infanticidal loss of their progeny.

We interpreted our results so that where possible, an out of home mated pregnant mare by behaving promiscuously she manipulates the home male's paternity assessment. If she has no chance to do that she may abort the current fetus. We cannot explain the mechanism yet, but speculate that this may be a mechanism actually activated by the mare [48].

3.2. The role of artificial insemination in pregnancy disruption

Artificial insemination (AI) is becoming increasingly used for initiation of pregnancy in domestic horses [71–73]; it was thus of interest to enquire whether disruption of pregnancies induced by AI occurs as frequently as after mating with a strange stallion away from home, and is affected by the same factors in the home social environment. Although an effect of natural versus artificial insemination was checked in the dataset analyzed in the first study, where it was found non-significant [48], a small proportion of AI in all pregnancies could affect the non-significant result. Therefore, we have collected more convincing amount of data for the next analysis.

Using PROC GLIMMIX for binary distribution (SAS version 9.4 with the mare's identity as a random effect), we analyzed 77 records of 51 different mares after AI, aged from 3 to 20 years,
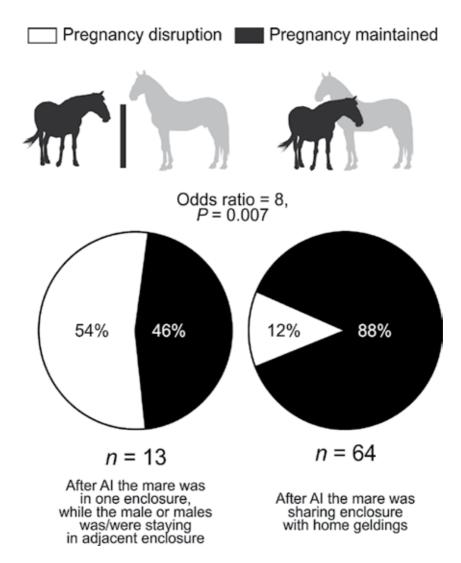
Figure 3. Proportion of pregnancy disruption according to social environment after AI of the mare according to social environment of the mare (reprinted from [59], permission for reuse provided by Oxford University Press).

giving birth between 0 and 13 foals, and bred between years 1984 and 2011. They belonged to 19 breeds and came from 21 individual breeders. As in the previous study [48], the probability of pregnancy disruption after AI depended on two effects only: social environment in which the mare was maintained after insemination (**Figure 3**) and the number of foals the mare had delivered in the past (**Figure 4**). As for natural mating the probability of failing reproduction was 8 times (odds ratio) more likely when the mare had no male company in her enclosure whereas stallions or geldings were present in an adjacent enclosure than when the mare was sharing the enclosure with geldings (in no case the mare was sharing the enclosure with a stallion). The results suggested that pregnancy disruption may equally be stimulated by the
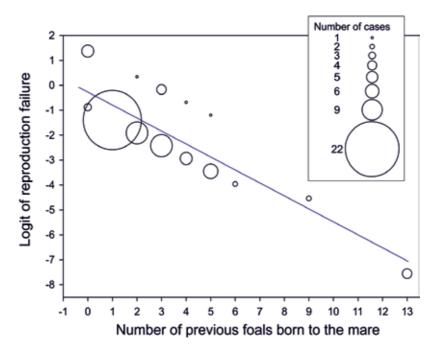
Figure 4. Probability of pregnancy disruption plotted against the number of previous foals born to the mare (reprinted from [59], permission for reuse provided by Oxford University Press).

social circumstances of the home environment in mares inseminated artificially as in mares mated naturally away from home. Present data thus support the prediction that the mare perceives conception after AI equally as mating with a new stallion.

3.3. The role of male free environment in pregnancy disruption

Since we published the results of the two above studies [48, 59], one additional questions had arisen. We did not consider the outcome if the pregnant mare was brought back to home farm containing mares only. Hence, in the final study on the domestic horse we tested the hypothesis that an incidence of a pregnancy disruption induced by a pregnant mare will be higher in a situation when a pregnant mare is in potential danger of the male's infanticide, compared to the situation when a pregnant mare is not in such a danger and/or can manipulate the male's paternity assessment by promiscuous mating [60].

For testing the hypothesis we used all the away-mated females from the previous study [48], extended them with additional 31 new records from pregnant mares mated away and then returned back to their home environment with one or more stallions and/or geldings ("home males"), and 34 records including situations with mares mated away and brought home to environment surrounded only by females herd mates. As in the initial study, all mares involved were positive in pregnancy testing carried out after mating. In total, there were 110 records of 75

different mares aged from 3 to 20 years, giving birth between 0 and 13 foals, and bred between years 1984 and 2011. They belonged to 21 breeds and came from 37 individual breeders [60].

Also in this last study on the domestic horse the data were analyzed with the aid of SAS (version 9.4) using a PROC GLIMMIX for binary distribution with the mare's identity as a random effect.

The probability of pregnancy disruption was significantly higher when the mare was in one enclosure, while the male or males stayed in adjacent enclosure in comparison to when the mare was sharing enclosure with home stallion and/or geldings or when the mare was sharing enclosure only with mares. In agreement with our prediction, the proportion of pregnancies which were blocked or disrupted was lowest when away-mated mares returned home to an environment containing only other mares (**Figure 5**). Perhaps surprisingly, the frequency of pregnancy disruption in this category did not, however, differ significantly from the situation when away-mated mares were sharing enclosure with home males and could manipulate the male's paternity assessment.
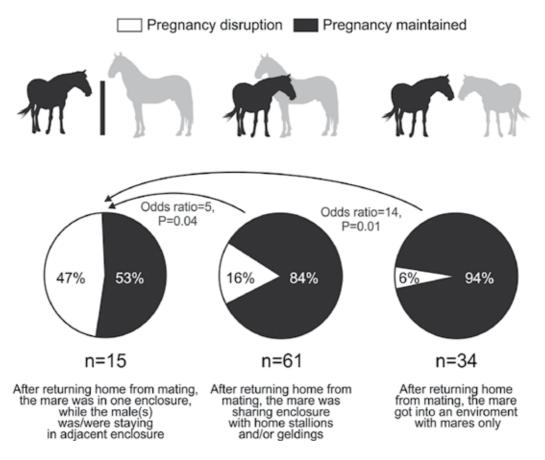

Figure 5. Proportion of pregnancy disruption according to social environment after returning to a home stable after mating and becoming pregnant elsewhere (reprinted from [60], permission for reuse provided by Elsevier).
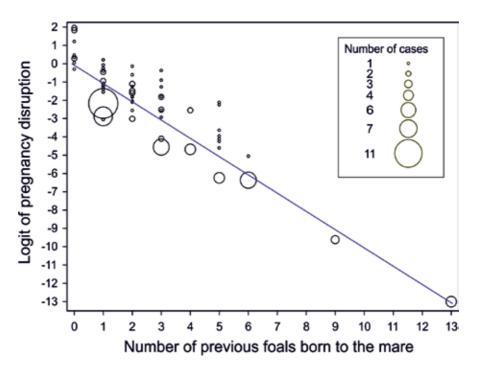
Figure 6. Probability of pregnancy disruption plotted against the number of previous foals born to the mare (reprinted from [60], permission for reuse provided by Elsevier).

When other factors were allowed to enter into the models only one other significant factor was found, the number of foals the mare had delivered in the past. With an increase in the number of foals delivered in her previous reproductive history the probability for the mare to disrupt pregnancy decreased (**Figure 6**).

4. Discussion

Taken together with the observation that the effect of social environment on pregnancy disruption was the same after artificial insemination, as with natural mattings away from home [59], strongly supports the original idea [48], that the primary factor modifying reproduction is a counterstrategy to the danger of potential infanticide [17]. In effect, whatever the method of insemination, once a mare is hormonally 'aware' of pregnancy, if she subsequently has no opportunity to mate with the home male (and thus confuse him about possible paternity), she will show increased likelihood of disrupting that pregnancy to avoid the costs of possible infanticide [compare also with 74]. An identical sociobiological origin of pregnancy failure has been recently shown in domestic dog *Canis lupus familiaris* [74]. In all three studies on domestic horse the probability of pregnancy disruption decreased with the number of foals a given mare had previously delivered (**Figures 2**, **4**, **6**). This fits well to the concept of

residual reproductive value, which is equal to an organism's reproductive value in the next age interval [75]. Each offspring thus becomes more valuable to a mother as the number of future potential offspring declines over her lifetime [75–77]. Such a result strongly supports the original idea [48], that the primary factor modifying reproduction is a counterstrategy to the danger of potential infanticide [17].

As yet we do not know the mechanism by which pregnancy disruption may be induced. Nonetheless, it is possibly triggered by stress associated with the presence of a new male [review in [78]]. Stress has been suggested in many studies to cause aborts in animals and humans [e.g., [79, 80–82]].

Results of the described studies support generally the presumption that sociobiological effect leading to pregnancy block and/or disruption may explain substantial part of the high incidence of domestic horse fetal loss reported over decades [6–8, 83, 84].

During our 20 years lasting research of male infanticide in equids we found that the incidence of this phenomenon is affected by social organization and social environment of individual species. Domestic horse represents the species with high incidence of male infanticide in contrast to other domesticated ungulates. Nevertheless, this phenomenon involves a various counter-strategies which might affect the reproduction as we demonstrated here. Thus, the detailed knowledge of the sociobiology might lead to practical implications in holding and breeding of individual domesticated species; in our case domestic horse.

5. Conclusions

In conclusion, after either mating away of home environment [48] or AI [59], horse breeders should place the pregnant mare into the facility with no stallion(s)/gelding(s) [60] or to an enclosure together with the mare(s) to prevent pregnancy block and thus to improve production as well as welfare of horses [48, 59].

Non-genetic, especially environmental and social factors should be routinely involved in studies investigating causes of pregnancy loss besides reproductive health and veterinary aspects. Still it is not the case either in studies of reproductive success [85–87] or welfare of breeding horses [88] even after our first report was published [48].

Acknowledgements

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The Use of Genetically Modified Organisms for Repopulation of Species of Commercial Importance in Aquatic Environment: Effects on Genetic Pool, Risks to Protected Areas and Policies for Their Proper Management

Maurilio Lara-Flores and Evelia Rivera-Arriaga

Abstract

In recent years, the reproduction of organisms through genetic engineering has been presented as an option for the repopulation of fish stocks of species that are at the limit or have passed their maximum sustainable exploitation. However, are the potential effects on genetic diversity known? The possible mutations? The risks to protected ecosystems? or Are there adequate policies and regulations for its management? This chapter aims to review the biological and population effects of the use of these organisms and the potential impacts they can cause to natural protected areas, as well as if there are adequate regulations or policies for their use. Finally, the authors give indicators for the sustainable integrated management of genetically modified organisms.

Keywords: genetically modified organisms (GMOs), aquatic environment, risks, policies, management

1. Introduction

Over the past 30 years, biotechnological developments have allowed scientists to alter the genetic make-up of bacteria, plants, and animals. At the beginning, the modifications had the purpose of carrying out basic research (the function of the gene and the mechanisms of genetic transfer); however, these techniques were quickly transformed into tools for use in

agricultural activities, since they allowed adding new characteristics to organisms in order to increase their capacities to be used in extensive monocultures (e.g., animals with a better neonatal survival or plants with herbicide resistance). The first genetically modified organisms (GMOs) were introduced in 1996, and currently around 200 million hectares are GMO crops worldwide [1, 2].

With the creation of the first genetically modified (GM) [3], various efforts have been made to modify the characteristics of farm animals to increase their production. Among the most studied characteristics are the increase in fertility [4], resistance to diseases [5], growth rate [6], and the reduction of the impact of activity to the environment [7].

An important source of protein for most people on the planet is products of marine and freshwater origin; these are mostly obtained through fishing; this activity causes serious consequences to the environment. The development of new fishing gears and the increase in the exploitation of aquatic species of commercial interest, together with the needs to supply the population with quality food, has prompted that in recent years this resource is at its maximum sustainable for its exploitation. Many populations of aquatic organisms are already overexploited, and their environmental and economic viability threatened in the medium term are considered. Genetically modified aquatic organisms are considered an option to increase the performance of aquaculture and as a means of repopulating natural stock; however, Italia use has generated a general concern for the possible impact on wild species with the consequent environmental damage [8].

Australia is the pioneer country in developing genetically modified aquatic organisms. Currently, the Maine Research in transgenic aquatic organisms has been developed in the United States, Canada, Cuba, China, and New Zealand. However, and despite biotechnological advances, many of these GMOs have only been used in aquaculture due the insecurity of the harmful effects on wild populations and the ecosystem [9].

This work aims to review the biological and population effects of the use of these organisms and their potential impact to natural protected areas, as well as if there are adequate regulations or policies for their use.

2. Genetically modified organisms

Genetically modified organism (GMO) refers to the organism in which there has been alteration of genetic material. GMOs are transgenic organisms into which desired DNA is inserted and incorporated with the help of *in vitro* techniques of genetic engineering.

The first transgenic animal was a mouse [10]. Rainbow trout [11] and goldfish [12] are the first transgenic production in aquatic species. Some of the significant species that today produced as GMO are Atlantic and Coho salmon, Tilapia species, catfish, medaka, and zebrafish.

For the development of GMO requires a series of steps to ensure its proper development. The steps can be summarized as follows: (a) identification of the gene of interest, (b) insolation of

the gene, (c) amplification of the gene, (d) insertion of the gene in appropriate plasmids, (e) the plasmid is cloned in bacteria to multiply it and be able to be injected, (f) the plasmid is inserted into the recipient tissue, normally fertilized eggs, (q) the gene is integrated into the receptor genome, (h) and finally the gene is expressed in the genome of organisms and is transferred to subsequent generations [9].

3. Ecological and genetic effect of transgenic aquatic species

Releases of genetically engineered organisms may directly or indirectly lead to changes in the natural environment. According to Williamson et al. [13], the environmental effects may be divided into three broad categories: (1) effects caused by the genetically engineered organism itself, (2) effects resulting from dispersal of genes from genetically engineered organism to other organisms in the environment, and (3) altered practice in the use of an organism because of the genetic modification.

The scale and frequency of introductions of transgenic aquatic species into a particular environment will greatly influence the degree of ecological risk involved [14]. According to Beardmore and Porter [14], the type and degree of ecological risk will vary depending on a number of factors: (a) the type of transgenic aquatic organisms, namely the overall phenotypic effect of the transgene, (b) the adaptive ability of the transgenic animals to the local environment, (c) the fitness of the transgenic fish, (d) the health of local populations, (e) the normal ecological role of the host species, (f) the potential for dispersal and persistence, and (g) the local environment itself.

The effects of GMO on wild ecosystems can be divided into two types [14, 15]:

a. *Intraspecific interactions:* the greatest ecological risk that GMOs can cause is the impact on the native populations of the ecosystem. The release to the environment of GMOs in order to increase their populations can cause a reproductive interaction between individuals of the same species, with what can cause a modification of the original genome of the species. If natural population comes to acquire the new genes, this can be translated into the loss of natural aptitudes and adaptations, for example, the resistance to diseases or the reduction in the anti-depredator response [15].

b. *Interspecific interactions:* an ecological risk associated with the introduction of GMOs to the environment is the impact on the aquatic communities with which it interacts. GMOs represent a risk within ecological niches and species. This is due to the competition between the species, mainly due to the possible aggressive behavior toward other individuals. This could lead to the destruction of target organisms of other species, the generation of new competitions for resources with other species, or the de-targeting of the specific habitat to one or several species, all this with the consequent imbalance of the ecosystem [14].

It is considered that GMOs cause a minimal risk to the environment, since many of these are modified for use under controlled conditions in farms or domestic use. On the other hand,

it is considered that GMOs that are used under controlled conditions will not survive in the environment without human help. However, it is necessary to evaluate the environmental risk that they can cause in case there could be a genetic exchange between the GMOs and the unaltered organisms that live naturally in the environment [16].

The effects caused by GMOs usually depend on the characteristics of the species, the environmental conditions, and the lifespan on the organisms. When evaluating the effects of GMOs, it should consider not only the effects in the same species but also the possible affectations to other species and the environment, this being the case that as GMOs and their genes can traverse in time and space. Therefore, it is essential to have all the documented information possible about environmental effects if you want to release a GMO, although it is practically impossible to predict such effects. On the other hand, it must be considered that GMOs can evolve for adaptation in the environment or for natural selection [17].

In the last years, research is being developed to create new transgenic fish in order to repopulate the natural water and for production purposes in farms. These investigations have been focused mainly to increase the speed of growth, size, resistance to diseases, and an increased survival in extreme habitats or in the face of climate change. In the environment, GMOs can be established permanently and irreversibly, with important effects on aquatic communities. Currently, mass gene transfer techniques such as lipofection, particle bombardment, and the electroporation of embryos and sperm cells are being developed and perfected in order to modify large batches of ovules [18]. The controversy about the release of GMOs to the environment has been widely discussed in the scientific and political environment [16, 19, 20]. In general, it has been observed that GMOs in the aquatic environment suffer from a greater predation [20], changes in eating habitats [21], a greater ability to compete for food [22], and better swimming skills [23].

To date, it is considered that the investigations to ensure the release of GMOs to the environment present sufficient sustenance to avoid damaging the environment; however, it is necessary to take into account that each GMO must be studied differently since its effects in the environment may vary [24, 25]. A clear example of the above has been observed in GMOs of plant origin where resistance to insects has been transferred from the cultivation areas to the wild areas causing significant losses in the production of honey [26, 27]. The effect that GMOs can cause in the environment is not known; however, it has been inferred that they could unbalance the ecosystem mainly at the trophic level due to the disappearance of species or the appearance of new diseases.

4. Management

Animal genetic resource for food provides an important element for sustainable development as well as for food security. However, this resource is poorly managed with dramatic consequences to local biodiversity, especially to the livestock sector. According to the Food and Agriculture Organization of the United Nations (FAO) [28] *"the biodiversity of the 35 or so animal species that have been domesticated for use in agriculture and food production is the primary biological capital for livestock development and is vital to food security and sustainable rural*

development. Many indigenous breeds, some of which are threatened with extinction, have characteristics such as resilience to climatic stress and resistance to diseases and parasites, which make them well adapted to local conditions, and which are of great potential importance to future livestock production." Also, FAO performed a balance of livestock which provided a comprehensive assessment and trends. It is important to enhance production conditions on the few breeds that are exploited worldwide, and that suffer from a high concentration of livestock development; otherwise, these may impose survival, production, and reproduction disadvantages for those animals. According to The State of the World's Animal Genetic Resources for Food and Agriculture [29], "twenty percent of documented livestock breeds are at a risk of extinction: 1500 of the 7600 breeds around the globe may be lost forever in the near future."

In relation to accidental introduction or the release of GMO in the wild, cause dispersion to other sites or even breed with individuals forms other wild populations. This fact continues to cause ecological concern globally, but there have been no published reviews of their effects in Mexico. Impacts in Mexico are therefore in need of a review, along with current legislation and guidelines relating to the introduction and control of such species.

To this day, it is impossible to assess the number of modified animal species that have been introduced to the wilderness in Mexico, both deliberately and accidentally, but only a small number of introduced GMO may suggest some degree of ecological impacts. However, to this point, it is difficult to consider that in Mexico, biodiversity may be negatively affected, and the potential effects of future climate change, and an increased number of genetically modified organisms may invade susceptible ecosystems. In this scenario, detrimental impacts if genetically modified organisms on native biota may occur through competition, predation, herbivory, habitat alteration, disease, and genetic effects (i.e. hybridization). In addition, there are potential effects on genetic biodiversity as well as species biodiversity.

Management measures for the control of potential negative effects on wild fauna might be more feasible if genetically modified animal organisms could be identified at an earlier stage of establishment. Nevertheless, it is very difficult to characterize modified organisms in the wild, and it is harder to foresee any impact that they may impose on ecosystems or wild populations. The difficulties of making general predictions suggest that every proposed organism introduction in the wilderness should be subject to rigorous ecological and sanitary characterization and risk assessment prior to introduction.

The nearly absence of Mexican legislation and guidelines developed on this matter to reduce impacts of genetically modified organisms into the wilderness is a critical gap that should be addressed as soon as possible. But still, to have this type of legislation is only part of the way toward ameliorating environmental impact. Many organisms already established in the wild might cause future problems. Illegal releases and escapes of genetically modified organisms may augment health and sanitary problems. While regulation of imports and releases is important, further enforcement of existing legislation and action against unlicensed releases is necessary.

Highly genetic manipulation and interbreeding, besides poor management, may result in antimicrobial resistance which poses a fundamental threat to human and animal health, development, local and national economy, as well as security. There are high levels of antimicrobial resistance due to overuse and misuse of antibiotics and other antimicrobials in both

● A No national AMR action plan

● B National AMR action plan under development or plan involves only one sector or ministry.

● C National AMR action plan developed that addresses human health, animal health and other sectors.

● D Multi-sectoral AMR action plan approved that reflects Global Action Plan objectives, with an operational plan and monitoring arrangements.

● E Multi-sectoral AMR action plan has funding sources identified, is being implemented and has monitoring in place.
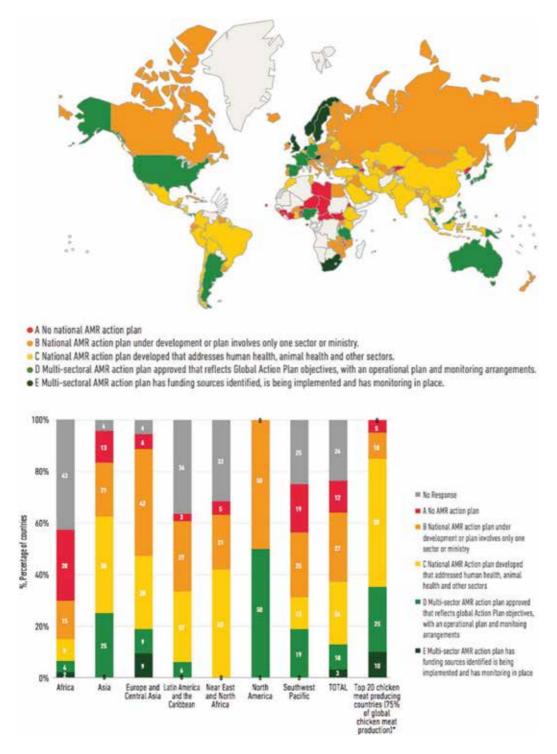

Figure 1. Country progress with the development of a national action plan on antimicrobial resistance as of May 29, 2017 [31].

humans and animals, including farmed fish and aquatic organisms, as well as residues of those drugs in the environment. The World Health Organization (WHO), FAO, and World Organization for Animal Health (OIE) have launched a Global Implementation Action Plan on Antimicrobial Resistance, with specific actions by May 2017 [30].

As of May 29, 2017, 147 countries had responded to the questionnaire (**Figure 1**). The overall findings show that countries have made significant progress in developing national action plans and implementing actions in the human and animal health sectors, in crop production, food safety, and the environment. Responses from countries also highlight areas where specific assistance and support are required [30].

From a new perspective, Laikre et al. [32] stated that "Large-scale exploitation of wild animals and plants through fishing, hunting and logging often depends on augmentation through releases of translocated or captively raised individuals." Such releases are performed worldwide in vast numbers. Augmentation can be demographically and economically beneficial but can also cause four types of adverse genetic change to wild populations: (1) loss of genetic variation, (2) loss of adaptations, (3) change of population composition, and (4) change of population structure. While adverse genetic impacts are recognized and documented in fisheries, little effort is devoted to actually monitor them. In forestry and wildlife management, genetic risks associated with releases are largely neglected.

Moreover, Mandel [33] in his review considered that the "regulation system of genetically modified organisms is far behind from biotechnology advances, which makes it passive rather than proactive about risks; difficult to adapt to new technology; and it is highly fractured and inefficient."

All these expose both the environment and the society to unnecessary risks that put difficult challenges to regulatory and management systems as well. These risks and inefficiencies include gaps in regulation, duplicative and inconsistent regulation, unnecessary regulatory expense, multiple agencies acting outside their areas of expertise, and unnecessary increase in the institutional, social, and environmental costs.

5. Conclusion

Ecological effects and the geographic ranges of organisms transcend political boundaries; therefore, we consider it essential to promote and achieve international coordination of risk assessment and regulation of biotechnology. Because the potential hazards of GMO are often environment-dependent, and ecosystems and biotas vary geographically and climatically, an organism that is safe in one country is not necessarily safe in other. Thus, both the commercial import and export and the inadvertent dissemination of GMO or their genes across political boundaries present special concerns that require cooperation and coordination. Special consideration must be given to the protection of rare genetic resources, such as the wild ancestors of domesticated species and threatened gene pools of other wild species. We urge local, state, national, and international cooperation in regulation, risk assessment, and risk management of the ecological effects of the introduction of GMO.

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Animal genetics is a field of science with important theoretical and practical significance in finding the answers for the actual problems of mankind. We hope that the readers will discover some new facts regarding the relationship between plasma proteins and boar semen freezability, the importance of some behavioral factors that affect reproduction in horses, and also an important chapter regarding the use of genetically modified organisms for the repopulation of species of commercial importance in aquatic environments, their effect on the genetic pool, risks to protected areas, and policies for their proper management.

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