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*Edited by Jaco Bakker, Manuel Gonzalez Ronquillo
and Rosa Maria Lino Neto Pereira*



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IntechOpen Book Series

Veterinary Medicine and Science

Volume 27

Aims and Scope of the Series

Paralleling similar advances in the medical field, astounding advances occurred in Veterinary Medicine and Science in recent decades. These advances have helped foster better support for animal health, more humane animal production, and a better understanding of the physiology of endangered species to improve the assisted reproductive technologies or the pathogenesis of certain diseases, where animals can be used as models for human diseases (like cancer, degenerative diseases or fertility), and even as a guarantee of public health. Bridging Human, Animal, and Environmental health, the holistic and integrative “One Health” concept intimately associates the developments within those fields, projecting its advancements into practice. This book series aims to tackle various animal-related medicine and sciences fields, providing thematic volumes consisting of high-quality significant research directed to researchers and postgraduates. It aims to give us a glimpse into the new accomplishments in the Veterinary Medicine and Science field. By addressing hot topics in veterinary sciences, we aim to gather authoritative texts within each issue of this series, providing in-depth overviews and analysis for graduates, academics, and practitioners and foreseeing a deeper understanding of the subject. Forthcoming texts, written and edited by experienced researchers from both industry and academia, will also discuss scientific challenges faced today in Veterinary Medicine and Science. In brief, we hope that books in this series will provide accessible references for those interested or working in this field and encourage learning in a range of different topics.

Meet the Series Editor



With more than 35 years of academic experience, Dr. Rita Payan-Carreira is now a Full Professor at the University of Évora (UE). She specializes in animal theriogenology, with a particular focus on the determinants of female fertility and fetal-maternal interaction during implantation. Dr. Payan-Carreira is dedicated to promoting a culture of collaboration and knowledge-sharing within the scientific community. In addition to her extensive publication record, she frequently collaborates with various publishing houses on peer-review activities. She further contributes to the scientific advancement of students and professionals by supervising academic theses and offering technical and scientific courses, including scientific communication, critical reasoning and discussion, and the ethical aspects of research.

Meet the Topic Editors



Jaco Bakker is an eager veterinarian actively involved in all research in laboratory animal science and medicine, whose aims are to improve veterinary care and welfare. Dr. Bakker is a member of the Association of American Primate Veterinarians, European Primate Veterinarians, the European Association of Zoo and Wildlife Veterinarians, the European Society of Laboratory Animal Veterinarians, and is a board member of the European Academy of Laboratory Animal Surgery.



Manuel González Ronquillo obtained his doctorate from the University of Zaragoza, Spain, in 2001. He is a research professor at the Faculty of Veterinary Medicine and Zootechnics at the Autonomous University of Mexico State. He received a Fulbright-García Robles fellowship for a postdoctoral stay at the US Dairy Forage Research Center in Madison, Wisconsin, USA, 2008–2009. He received grants from Alianza del Pacífico for a stay at the University of Magallanes, Chile, in 2014, and from the Consejo Nacional de Ciencia y Tecnología (CONACyT) to work in the Food and Agriculture Organization's Animal Production and Health Division (AGA) in Rome, Italy, in 2014–2015. He has collaborated with researchers from various countries and has published over 90 journal articles. He teaches various degree courses in zootechnics, animal nutrition, animal health, animal production, and agricultural sciences and natural resources. Dr. Ronquillo's research focuses on evaluating sustainable animal diets (StAnD) using native regional resources, reducing carbon footprints, and applying meta-analysis and mathematical models to understand animal production better.



Rosa Maria Lino Neto Pereira is a full researcher at the Genetic Resources and Biotechnology Unit of the National Institute of Agriculture and Veterinary Research. She is the head of the Reproduction Department and the Embryology Laboratory, and a lecturer in Theriogenology at the Veterinary Medicine Faculty. She has over 30 years of experience working in embryo and gamete cryopreservation for research and animal genetic resources (AnGR) conservation, leading research teams and projects. Rosa Pereira is also a member of national and international networks (AniDop, ERFP – FAO, EUGENA) for the conservation and promotion of AnGR. Her current research interests include: Portuguese AnGR characterization and in situ/ ex situ conservation; identification of mechanisms underlying fertility or infertility; and improvement of the efficiency of reproductive biotechnologies.

Contents

Preface	XIII
Section 1	
Animal Science	1
Chapter 1	3
The Innate and Adaptive Immune System of the Common Bed Bug, <i>Cimex lectularius</i> : Current Knowledge and Research Opportunities <i>by Sanam Meraj and Gerhard Gries</i>	
Chapter 2	23
A Comprehensive Review of Non-Infectious Shell Disorders of Chelonia <i>by Remco A. Nederlof, Doortje W. van Reekum, Job B.G. Stumpel and Jaco Bakker</i>	
Section 2	
Animal Nutrition	45
Chapter 3	47
The Role of Phytogenic Feed Additives in Modulating Poultry Nutritional Physiology and Genomics <i>by Hasan Hüseyin Ipçak</i>	
Chapter 4	79
Assessment of the Impact of Ungulate Browsing on Tree Regeneration <i>by Andrea Doris Kupferschmid, Raphaël Greilsamer, Peter Brang and Harald Bugmann</i>	
Chapter 5	99
Application of Vermicompost Fertilizer in Aquaculture Nutrition: Review <i>by Sonnia Nzilani Musyoka and Rita Nairuti</i>	

Section 3	
Animal Reproductive Biology and Technology	113
Chapter 6	115
Cryopreservation of Oocyte in Livestock: Principles, Techniques and Updated Outcomes	
<i>by Thivhilaheli Richard Netshirovha, Vhahangwele Makumbane, Lerato Deirdre Sehlabela, Zwivhuya Constance Raphalalani and Masindi Lottus Mphaphathi</i>	

Preface

Bed bugs and chelonians have in common that they have existed on our planet for millions of years. Bed bugs have existed for over 100 million years; they have co-existed with dinosaurs. Chelonians, a group that includes turtles and tortoises, have existed for about 280 million years. Interestingly, despite their ‘age’, there are still knowledge gaps concerning both. The “Animal Science” section provides updates on advances in knowledge of bed bugs and chelonians, which are continually evolving and driven by technological advancements and improved understanding of anatomy, ecology, immunology, and physiology. Despite their ‘age’, it is clear that many components still await discovery.

Jaco Bakker

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Topic Editor: Animal Science

This section addresses topics related to animal nutrition, including the effects of phytogenic feed additives (PFAs) on performance, gut health, microbiota, oxidative stress, the antioxidant defense system, gene expression, and protein production in poultry. These additives are considered an alternative to synthetic growth promoters to increase productivity and facilitate the transition to a more sustainable, natural production system. The impact of ungulate browsing on tree regeneration is also analyzed, as are the time required for trees to grow beyond the reach of herbivores, potential changes in growth rate rankings among selected species, and a possible delay in tree response after grazing. Finally, the application of vermicompost fertilizer in aquaculture nutrition is reviewed. Vermicompost has been recognized as a potential pond fertilizer due to its superior nutritional quality (up to 5 times higher), microbial content, and ready-to-use formulation.

Manuel Gonzalez Ronquillo

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Topic Editor: Animal Nutrition

Reproductive biology and related technologies are attracting increasing interest from students, technicians, producers, and broader society. This interest encompasses medical, social, and economic aspects, as well as a dichotomy between the prevention and treatment of infertility and the control of reproduction. Technological advances in many areas of biomedical science and reproductive biotechnologies have opened up new possibilities for improving genetic gain, preserving germplasm, or resolving infertility cases, thereby increasing reproductive efficiency and animal productivity, and conserving animal genetic resources. This section presents and discusses examples of these technological advances.

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Topic Editor: Animal Reproductive Biology and Technology

Section 1

Animal Science

The Innate and Adaptive Immune System of the Common Bed Bug, *Cimex lectularius*: Current Knowledge and Research Opportunities

Sanam Meraj and Gerhard Gries

Abstract

The common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae), is a blood-feeding ectoparasite of vertebrates, primarily humans. In contrast to many other hematophagous arthropods, such as kissing bugs, mosquitoes, sandflies, and ticks that intermittently seek blood meals from vertebrate hosts, *C. lectularius* does not vector disease-causing pathogens and parasites to their human hosts. In this review, we summarize currently known immune responses by *C. lectularius*, and propose worthy research topics. Challenged by microbe ingestion or infection, *C. lectularius* mounts cellular immune responses such as phagocytosis of bacteria, as well as humoral responses such as secretions of antimicrobial peptides into the hemolymph. The functional immune system of the hemimetabolous *C. lectularius* resembles that of holometabolous insects but exhibits distinct deviations, including a sparser immune repertoire, the production of DNA nets by cells in response to pathogen invasions, and reproductive immune anticipation in the context of sexual reproduction (traumatic insemination). Many components of the *C. lectularius* immune system still await discovery, including the receptor molecules and immune pathways involved in antiparasitic and antiviral immune responses. Why *C. lectularius* does not vector pathogens to human hosts is hardly understood. Potential explanations include upregulated antimicrobial peptide activities that help eliminate invading pathogens.

Keywords: *Cimex lectularius*, bed bug, innate immunity, antimicrobial peptides, *Wolbachia*, traumatic insemination

1. Introduction

The insect immune system has two parts: the innate (general) immune system and the adaptive (specialized) immune system. The innate immune system is the first line of defense against invading pathogens and includes cellular and humoral responses that help detect and eliminate invasive pathogens without harming the

insects' own obligate microbiota [1, 2]. The adaptive immune system draws on immune memory from previous pathogenic attacks and prepares for more effective immune responses to subsequent infections [3]. The adaptive immune system enables pathogen-specific responses and relies on cells with specific receptors that recognize pathogens. The insects' true adaptive immune responses are not as elaborate as those of their mammalian counterparts, but in response to prior microbial insults insects display immunological priming which heightens humoral and cellular responses to subsequent insults and thus increases the likelihood of insect survival [3, 4].

Most of our knowledge about insect immunology stems from research on holometabolous model insects, such as the vinegar fly *Drosophila melanogaster* and the yellow fever mosquito, *Aedes aegypti*. Yet, the immunology of other insect taxa may be different [5–7]. For example, the immune pathways of hemimetabolous insects seem to have anomalies and to be sparser than those of holometabolous insects [8]. In this review, we focus on the immune system of the hemimetabolous common bed bug, *C. lectularius* (Hemiptera: Cimicidae), which is an obligate hematophagous ectoparasite, primarily of humans. We highlight immune responses of *C. lectularius* that deviate from those of the holometabolous immunological models.

The immune system of *C. lectularius* is thought to be unique and more efficient than that of most other insects. The extraordinary immune system of *C. lectularius* may have evolved in response to its peculiar mode of sperm transfer, known as hypodermic or traumatic insemination [9]. During traumatic insemination, the male pierces the female's abdomen with his aedeagus (penis), a sickle-shaped paramere, and injects his sperm and accessory gland fluids through the wound into her abdominal cavity (hemocoel). The sperm then diffuses through the female's hemolymph, eventually reaching her ovaries where fertilization takes place [9–11]. Traumatic insemination occurs more frequently than necessary for egg fertilization [12] and is detrimental to the female's health. It creates an open wound which is costly to repair and susceptible to infection. The injection of both sperm and ejaculatory fluids into the hemocoel may trigger immune responses by the female. As females commonly get mated after a blood meal, they anticipate traumatic insemination ('reproductive immune anticipation'), using blood feeding as a cue to 'prophylactically' produce lysozyme-like activity (LLA) in their spermatheca [13], a pair of sperm-receptacles which may help provide hygienic protection against bacteria [11]. Copulatory wounding introduces pathogens to both male and female bed bugs [14].

Many hematophagous insects or arthropods, such as fleas, kissing bugs, mosquitoes, sandflies, tsetse flies, and ticks, that intermittently seek blood meals from vertebrate hosts, competently vector disease-causing pathogens and parasites, including bacteria, fungi, protozoa and viruses [15]. These pathogens or parasites cause debilitating diseases (e.g., Chagas, dengue, leishmaniasis, lymphatic filariasis, malaria) in humans [16] and millions of deaths each year [17]. In contrast, *C. lectularius* does not naturally (outside laboratory settings) transmit pathogens or parasites to vertebrate hosts [18–21], even though more than 40 disease-causing organisms have been recovered in or on tissue from *C. lectularius* [19]. There is no definitive knowledge as to why *C. lectularius* lacks vector competence, but potential explanations lie with the innate immune responses of *C. lectularius*. Here, we review aspects of immunity studied in *C. lectularius*, summarize findings about the *C. lectularius* immune system, and provide explanations as to why *C. lectularius* lacks vector competence outside controlled laboratory conditions.

2. The innate immune system of *C. lectularius*

As shown in other insects, the innate immune system of *C. lectularius* comprises multiple components that work in concert. These components are grouped in three broad categories (**Figure 1**): (1) physical barriers such as the cuticle and epithelia; (2) cellular responses including the elimination of pathogens through phagocytosis, nodulation, and encapsulation; and (3) humoral responses comprising the biosyntheses of antimicrobial peptides (AMPs) and lectins, and the activation of the prophenoloxidase (PPO) cascade. In all insects studied thus far, pathogen-associated molecular patterns (PAMPs) on the surface of pathogens are recognized by pattern receptor proteins (PRPs) as host sensors that initiate cellular and humoral responses.

2.1 Physical barriers

The insect integument (cuticle) serves as an exoskeleton, physical barrier, and first line of defense, preventing water loss, penetration, and invasion of chemicals and pathogens that interfere with the insect's homeostasis [22]. Prompted by surface injury, the outermost epicuticle produces AMPs and thus contributes to innate immune defense [23]. The cuticle is conserved across arthropods [24, 25]. It is made of chitin fibrils implanted in a matrix of proteins, lipids, and N-acylcatecholamines [24, 25]. Cuticular linings cover all external epithelial tissues, as well as the insects' foregut and hindgut.

The cuticle of *C. lectularius* differs from that of other insects in that it has two (instead of one) physical barriers with distinct regions of temperature-sensitive, lipid-based physico-chemical properties that protect against penetration of hydrophilic molecules [26]. The outer barrier constitutes cuticle protruding nano- and macrostructures [27], whereas the inner barrier consists of lipophilic waxes and free cuticular hydrocarbons [27–29]. These double barriers may provide fail-safe protection against penetration by external pathogens.

The peritrophic membrane covers and protects the midgut lumen of insects. It functions as a physical barrier against abrasive food particles and digestive pathogens [30], and it prevents various pathogens from readily passing into the hemolymph. In most hemipterans, the peritrophic membrane is absent and, instead, an extracellular lipoprotein membrane, the perimicrovillar membrane, protects the midgut epithelium. In *C. lectularius*, the functional role of this perimicrovillar membrane has not specifically been studied but its importance in hemipteran immunity has been discussed, and a protective function has been suggested for various hemipterans [31]. If microorganisms still pass through the midgut barrier into the hemolymph, humoral and cellular immune responses are activated [32].

2.2 Cellular immunity

2.2.1 Types of hemocytes in insects and *C. lectularius*

Hemocyte immune responses greatly vary among the many insect species, and we are just beginning to understand these variations [4]. However, in most species studied thus far, four frequent cellular immune responses have been described: nodulation, encapsulation, melanization, and phagocytosis. Cellular immune responses take effect immediately after pathogen invasions of the hemocoel, whereas humoral

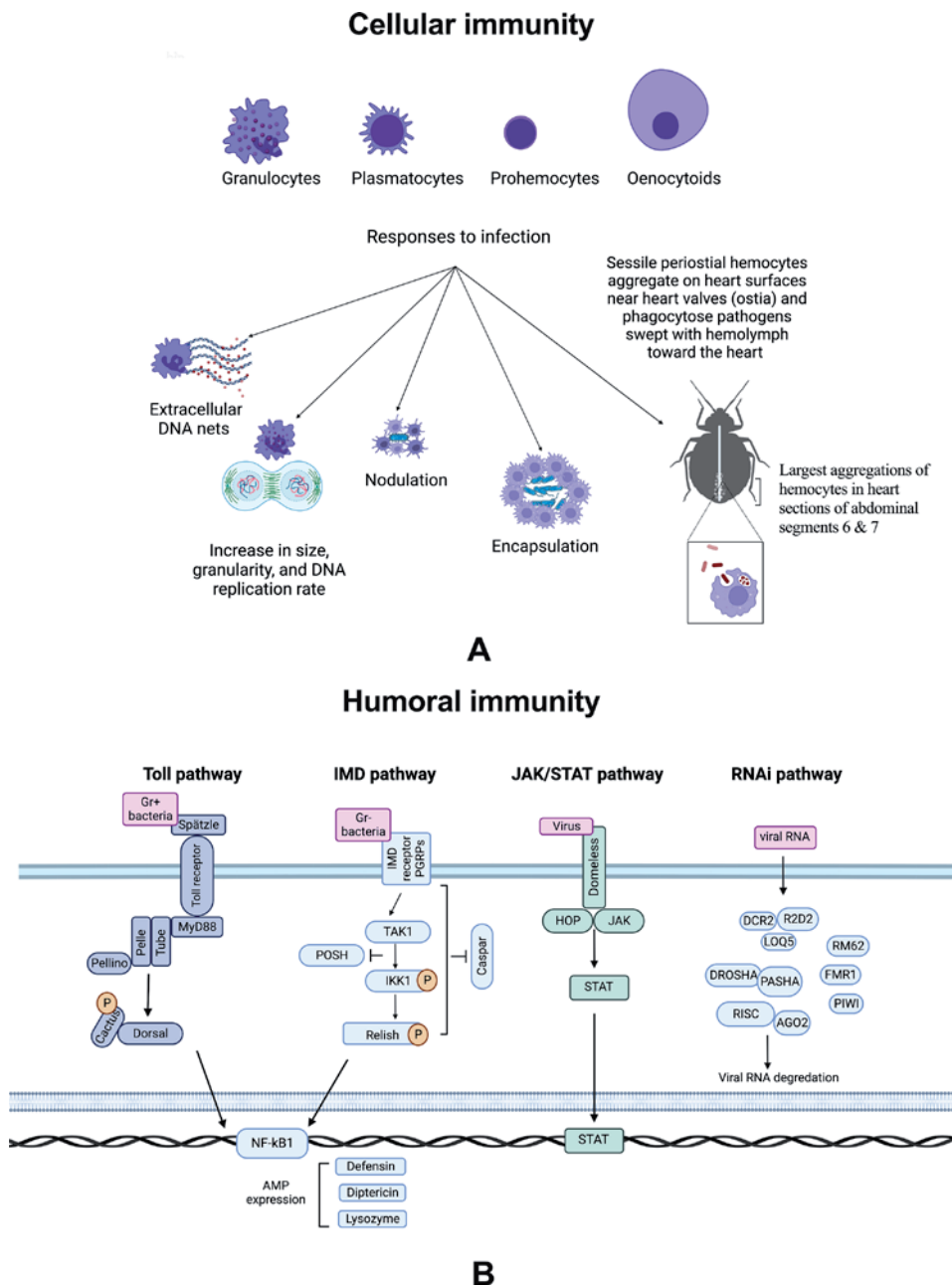


Figure 1.

Cellular and humoral immune responses of the common bed bug, *Cimex lectularius*. **A.** Cellular immune responses involve immune cells (hemocytes), such as granulocytes, plasmatocytes, prohemocytes and oenocytoids [21], that engage in phagocytosis, nodulation, and encapsulation of pathogens, and that form extracellular DNA traps in response to pathogen infections [21, 45]. **B.** Illustration of the orthologs of the Toll, IMD, JAK/STAT, and RNAi pathways functional in *C. lectularius* and portrayed based on general immune pathways in insects [5]. This figure was created with BioRender.com.

responses appear several hours later [33]. Cellular immune responses are carried out by specialized immune cells, the sessile and circulating hemocytes. Sessile hemocytes are associated with specific tissue and adhere to internal organs such as the insect's

fat body and digestive tract, whereas circulating hemocytes are moving in the hemolymph [33]. The density of hemocytes in the hemolymph varies during the life of an insect and in response to pathogen invasions [33]. Like human immune cells, insect hemocytes recognize foreign particles and distinguish self from non-self during cellular immune responses [34].

Insect hemocytes are classed according to their form or function but classes are well characterized only in *Drosophila* by establishment of molecular markers [35]. Not all hemocyte types occur in all insects but at least eight classes of insect hemocytes are characterized to date: prohemocytes, plasmatocytes, granular cells (granulocytes), coagulocytes, crystal cells, spherulocytes, oenocytoids, and thrombocytoids. Prohemocytes are circular and contain a large nucleus. They are thought to be equivalent to stem cells and are the smallest of all hemocytes. In insects, plasmatocytes, along with granulocytes, are the most common cell type, performing distinct immune functions. Plasmatocytes contain lysosomal enzymes in their cytoplasm and their major function is phagocytosis [36]. Granulocytes are characterized by the presence of secretory vesicles (granules) in their cytoplasm and are involved mainly in phagocytosis in lepidopterans [36–38]. During the immune process of degranulation, granulocytes release antimicrobial, cytotoxic or other immune molecules from their granules. In lepidopterans, plasmatocytes and granulocytes are involved in most cellular defense responses [37], whereas in *Drosophila* plasmatocytes and lamellocytes mount equivalent responses [37]. In lepidopterans, oenocytoids are major producers of the enzymes that are required for melanization [37, 38]. Coagulocytes contribute to hemolymph coagulation (clotting) [39]. Spherulocytes are characterized by conspicuous spherical inclusions (spherules) in their cytoplasm. They have different shapes and sizes and perform different functions such as coagulation and transport of cuticular materials [39–41]. Finally, crystal cells are present in only some insects. In *Drosophila*, they contain the key enzyme PPO that mediates the biosynthesis of melanin (melanization; see below) [42].

There are only a few reports on hemocytes in the *Cimicidae*. *In vitro* studies of circulating hemocytes in nymphal *Cimex rotundatus* revealed six types of hemocytes: prohemocytes, plasmatocytes, granulocytes, spherulocytes, oenocytoids and thrombocytoids (a variant form of granulocytes and a functional analogue of coagulocytes) [43]. Plasmatocytes and granulocytes are considered the major circulating cells in *C. rotundatus* nymphs and adults, respectively [43]. In *C. lectularius*, circulating hemocytes were classed based on their size, morphology, and staining properties [21]. Granulocytes and plasmatocytes were the most abundant cells, and granulocytes, plasmatocytes, prohemocytes and oenocytoids – but not thrombocytes – were found in adult *C. lectularius* (**Figure 1A**) [21], with hemocyte granularity in females being significantly greater than in males [21]. Giant cells such as oenocytoids are present in *C. lectularius* but are rare in triatomine bugs [44]. Furthermore, experimental infection of *C. lectularius* and yellow fever mosquitoes, *A. aegypti*, with the Gram-negative bacterium *Escherichia coli* induced migration of hemocytes to perioistial surfaces of the heart, thereby amplifying immune responses [45]. *Cimex lectularius* infected with *E. coli* had 2-times more heart-associated hemocytes than untreated control specimens [45]. In the innate immune system of *C. lectularius*, the role of each hemocyte type in response to different pathogens has not been specifically characterized. Determining the specific hemocytes associated with melanization, encapsulation, and nodulation would greatly enhance our understanding of the *C. lectularius* immune system.

2.2.2 Degranulation and size increase of *C. lectularius* hemocytes in response to infection

Degranulation, increase of hemocyte size, and greater DNA replication rates, are hallmarks of immune cell activation [32, 46–48], and were observed in male and female *C. lectularius* [21]. Sessile and circulating hemocytes of *C. lectularius* underwent cell divisions in response to bacterial exposure [49]. Moreover, hemocytes of male and female *C. lectularius* transitioned from the G0/G1 phase to the S- and G2-phase when exposed to heat-killed *E. coli* [21], thus confirming the activation of cell division as an immune response. All observations combined provide evidence for immune cell activation in *C. lectularius*.

2.2.3 Phagocytosis

During the process of phagocytosis, hemocytes engulf targets such as bacteria, yeast, and apoptotic bodies, and even small experimental artifacts such as synthetic beads or India ink particles [50, 51]. In insects, it is the plasmatocytes and granulocytes that serve a phagocytic function [4], but in mammals the phagocytic neutrophils serve that function [52]. In silkworms, *Bombyx mori*, activation of the PPO cascade is necessary for granulocytes to attach to pathogens and to initiate phagocytosis [53, 54]. After an immune challenge, only the plasmatocytes of the triatomine bug *Rodnius prolixus* engaged in phagocytosis [55], whereas several (unspecified) types of *C. lectularius* hemocytes phagocytosed heat-killed *E. coli* [21]. Sperm of male *C. lectularius* that failed to travel to the periphery of the female's spermatheca is phagocytosed by hemocytes or attacked by lysozyme [56]. Furthermore, experimental injection of *C. lectularius* with *E. coli* induced aggregation of hemocytes on the tubular heart and rapid phagocytosis of *E. coli* circulating with the hemolymph [45]. Consistent with hemocyte aggregation patterns reported in both *Rhodnius prolixus* [57] and the boxelder bug, *Leptocoris trivittatus* [58], *C. lectularius* hemocytes aggregate mainly on the section of the heart that resides in abdominal segments 6 and 7, near the mesospermatheca in abdominal segments 5 and 6, where traumatic insemination takes place [12, 45]. The *C. lectularius* genome contains candid phagocytosis-related genes [5] that still need thorough exploration.

2.2.4 Melanization, nodulation and encapsulation

Melanization, the biosynthesis of melanin, is a prominent immune response in insects and arthropods [38, 40, 69] and serves multiple roles, including the encapsulation of pathogens and parasites, wound healing, and the production of cytotoxic chemicals that kill invading microorganisms [38, 45, 59–63]. Melanization contributes to the elimination or killing of bacteria, fungi, protozoan parasites, nematodes, and other organisms that have invaded an insect body. Melanization involves multiple components, including PRPs as host sensors that initiate cellular and humoral responses, serine protease cascades, and a phenoloxidase enzyme. Ultimately, melanin surrounds and sequesters an invading pathogen. The pathogen's death is thought to be caused by oxidative stress via reactive oxygen species or by starvation, achieved by isolating the pathogen from nutrient-rich hemolymph [60, 61]. In the spermatheca and hemocoel of female *C. lectularius*, melanotic and cellular encapsulation in response to heat treatment was measured [64].

Nodulation (the aggregation of hemocytes around microorganisms) is the main insect defense response to eliminate large cohorts of bacteria that have invaded the hemolymph. In the process of nodulation, an overlapping sheet of hemocytes forms and surrounds pathogens [36]. Nodulation may further involve melanization and the activation of the enzyme PPO [38]. The end products of the phenoloxidase cascade are melanin and toxic byproducts such as free oxygen species, phenols and quinines, that may kill pathogens or prevent further growth [38, 63].

Encapsulation resembles nodulation but targets larger objects in the hemolymph, such as parasitoid eggs and larvae as well as nematodes [65]. Cellular encapsulation is common in dipterans, occurs with or without hemocyte assistance, and always involves PPO activation [59]. Humoral encapsulation, in contrast, occurs mainly in lepidopterans and can take place without melanization [65]. The volume of encapsulating cells and the degree of melanization in the spermatheca and hemocoel of *C. lectularius* were measured in response to *Wolbachia* load [64].

Genes encoding encapsulation and nodulation processes as well as melanization/prophenoloxidase (PPO) pathways are conserved across many insect taxa and have also been identified in the genome of *C. lectularius* [5]. An increase in two PPO-like transcripts was shown in *C. lectularius* hemocytes exposed to heat-killed *E. coli* [21]. The activation of these genes should also be studied in response to other pathogens.

2.2.5 Extracellular DNA traps

As only recently shown, *C. lectularius* hemocytes form extracellular DNA traps in response to pathogenic infection [21]. These traps resemble the mammalian neutrophil extracellular traps (NETs) [66]. In mammals and some insects, the controlled release of chromatin (complex of DNA and protein) from the nucleus of hemocytes provides scaffolds, with hemocyte-mediated defense factors, that trap and eliminate microorganisms [67, 68]. Extracellular DNA traps have been found in only a few insect species. The effect of these DNA traps on specific pathogens, and the immune pathways that are activated in response to these pathogens need to be explored.

3. Humoral immune responses by *C. lectularius*

Insect humoral defenses include the biosynthesis of AMPs [69, 70] and reactive intermediates of oxygen or nitrogen [71, 72] as well as activation of complex enzymatic cascades that regulate coagulation or melanotic encapsulation of parasites and pathogens [73, 74]. PRPs of insect hosts detect PAMPs on the surface of pathogens and activate nuclear factor-kappa B (NF- κ B) transcription factors that are involved in immune responses such as AMP expression. Pathways involved in this mechanism include the immune deficiency (IMD) pathway, the Toll pathway, and the JAK/STAT pathway [38].

The genome of *C. lectularius* contains strong candidate genes for recognition molecules, which are the key members of the Toll, IMD, Jak/STAT and RNA interference (RNAi) immune pathways that have been thoroughly characterized in model insects (**Figure 1B**) [5]. Compared to holometabolous insects, the immune repertoire of *C. lectularius* appears sparser for Toll, IMD, and Jak/STAT pathways [8], and only a few recognition molecules and AMPs have been identified. However, this paucity of immune components reported for *C. lectularius* may be attributed to inadequacies

of standard automated genome annotation programs. In analyses of hemipteran transcriptomes, many immunity-related genes may have been overlooked [75] due, in part, to the presence of multiple short introns and sequence divergence between holo- and hemimetabolous insects [75].

Furthermore, tissue- and pathogen-specific upregulation of immune pathways in *C. lectularius* has only recently received attention. In *C. lectularius*, upregulation of effector genes in Toll and IMD pathways has been studied only in hemocytes in response to *E. coli* infection, revealing no significant upregulation of most of the key components of these pathways [21]. In addition, NF- κ B signaling inhibitors – that inhibit Toll and IMD pathways with high specificity in other organisms – were ineffective in *C. lectularius*, and only one inhibitor (IKK16) of NF- κ B signaling significantly enhanced the rate of *C. lectularius* mortality from oral infections with the bacterium *Pseudomonas entomophila* [76]. Despite these anomalies, AMPs normally regulated by the IMD pathway are still expressed in *C. lectularius*, suggesting that the IMD and Toll pathways are functional [77, 78]. Further research on *C. lectularius* is needed to study pathogen-specific responses of multiple immune pathways and potential cross-talk between pathways, as shown in triatomine bugs and other insects [79].

3.1 Lysozyme-like activity (LLA), lysozymes, antimicrobial peptides (AMPs) and AMP activity in *C. lectularius*

Immune responses such as LLA and AMP activity have been studied in the midgut, hemolymph, fat body, and reproductive organs of female and male *C. lectularius* (Table 1).

Tissue	Key findings/topics studied	References
Entire body	Lysozyme-like genes, defensin-like peptides, dipteracin-like peptides in males	[5]
Entire body	Antimicrobial peptide activity against Gram-positive and Gram-negative bacteria 24 h after blood ingestion or bacterial immune challenge in males and females	[81]
Saliva	Lysozyme peptides	[80]
Midgut	Antimicrobial peptide activity against Gram-positive and Gram-negative bacteria 24 h after blood ingestion or bacterial immune challenge in males and females	[81]
RoB ^a	Antimicrobial peptide activity against Gram-positive and Gram-negative bacteria 24 h after blood ingestion or bacterial immune challenge in males and females	[81]
Spermalege	Predictable infradian feeding cycles serve females as cues to impending immune insults by males during traumatic insemination	[13, 14]
Ejaculate & sperm	Lysozyme-like activity in males	[80]
Hemolymph	Lysozyme-like activity in males and females	[80]

^aRoB = Rest of Body (containing bodies minus the heads and midgut tissues).

Table 1.

List of key findings, topics studied, and body tissues analyzed in immune response experiments with female and male bed bugs, *Cimex lectularius*.

3.1.1 Lysozyme-like activity and lysozymes

Immune response-induced LLA- and AMP-activities in the copulatory organ (spermalege) of female *C. lectularius* are well documented. With predictable infradian feeding cycles that serve as cues to imminent immune insults by males during traumatic insemination, females increase their LLA-activity in the spermalege [13]. Similarly, in response to bacterial transfers from males to females, females increase their LLA-activity [14]. While females receive bacteria from males, they also transfer bacteria to males [14]. There are at least three indications for mutual sexual transmission of bacteria between mating partners: (1) copulation increased the similarity of bacterial communities in male and female reproductive organs; (2) mated bed bugs harbored bacteria that were found in non-mated opposite-sex bed bugs but not in non-mated same-sex bed bugs; and (3) bacterial communities showed a high turnover between non-mated and mated bed bugs, suggesting a mating-induced replacement of bacteria [14]. Furthermore, the saliva of *C. lectularius* contains lysozyme and peptides with presumed antimicrobial activity [80]. LLA-activity was also shown in the sperm, seminal fluid and hemolymph of male *C. lectularius* [80]. Despite these reports of LLA activity in *C. lectularius*, and evidence for lysozyme-like genes in the genome of *C. lectularius* [5], the upregulation of transcripts in various tissues of *C. lectularius* has hardly been studied.

3.1.2 Antimicrobial peptide activity and defensins

There was time-dependent AMP activity in midgut and rest of body (RoB) samples (containing bodies *minus* the heads and midgut tissues) obtained from *C. lectularius* that were blood-fed or immune-challenged with Gram-positive and Gram-negative bacteria [81]. AMP activity was significantly upregulated in both midgut and RoB samples taken 24 h post infection with Gram-positive and Gram-negative bacteria, or 24 h post blood-feeding. The stronger immune response by females than males within 24 h of a blood meal [81] is likely an adaptation to battle immune insults caused by traumatic insemination. Cross-taxa studies revealed that blood-feeding induced stronger whole-body antimicrobial activity in female *C. lectularius* than in female *A. aegypti* [81], consistent with larger *E. coli*-induced aggregations of phagocytic hemocytes on the hearts of *C. lectularius* than on the hearts of *A. aegypti* [45].

In insects, AMPs are upregulated in response to bacterial exposure or blood feeding, and are produced in specific body tissues, including the intestinal tract, fat body cells, tracheae and hemocytes [82]. Of all identified AMP groups, only a few AMP-like genes/peptides and their variants have been identified from the genome or transcriptome of *C. lectularius* [5]. These include defensin-like peptides, dipteracin-like peptides and lysozymes.

Defensins are small, variable cationic arginine-rich peptides [83]. They are ancient natural antibiotics with strong antimicrobial activity against a range of microorganisms [83]. More than 300 defensins have been identified but they are not specific to insects. The *C. lectularius* defensin (CL-defensin) is the only AMP that has been characterized for its effectiveness against human skin microbial flora including Gram-positive and Gram-negative bacteria [84]. Defensin-like transcripts increased in *C. lectularius* hemocytes in response to immune challenges with Gram-negative *E. coli* [21]. Dipteracin-like peptides in the genome of *C. lectularius* [5] belong to a family of glycine-rich antibacterial peptides that are effective against Gram-negative bacteria [83]. The presence of dipteracin-like transcripts has been illustrated in *E. coli*-infected

C. lectularius hemocytes [21]. Tissue- and bacteria-specific upregulation of defensin-like and dipteracin-like genes needs to be studied further in *C. lectularius*.

4. Immunity-related effect of symbionts in *C. lectularius*

The microbiome is defined as the collection of microorganisms, their genomes, and the surrounding environment [85]. Within the last decade, the importance of the microbiome has become increasingly evident, both in humans [86] and in insects [87]. Insect immunity can no longer be considered in isolation of the insect microbiota and symbionts. The endosymbiotic relationship of microorganisms living inside a dissimilar organism (host) ranges from mutualism (both host and symbionts benefit from the relationship) to parasitism (symbionts benefit to the detriment of the host) [88]. Bacterial endosymbionts can aid the immune system of insects in response to invading pathogens and can increase survival and reproduction of their insect hosts. Endosymbionts induce immune priming for subsequent pathogen invasions [89–92], produce AMPs [90], and outcompete invading pathogens for resources [91].

The midgut microbiota of mosquitoes is a determinant factor for their vectoral capacity or susceptibility [92]. For example, mosquitoes harboring the Gram-negative bacterium *Serratia odorifera* in their midgut are more susceptible to infections with the Chikungunya virus that causes Chikungunya fever in vertebrates [93], and mosquitoes harboring the fungus *Talaromyces (Penicillium) marneffei* are more susceptible to the Dengue virus that causes Dengue fever in vertebrate hosts [94]. Compared to other indoor pest insects, the microbiome of *C. lectularius* is unusually sparse [95], and its contribution to host immunity is largely unknown. Proteobacteria, an unclassified γ -proteobacterium, intracellular *Wolbachia* bacteria, and a *Pectobacterium* accounted for 98–99% of all bacteria present in the midgut of *C. lectularius* [96, 97]. Comparing the microbiota of kissing bugs, mosquitoes and bed bugs would provide insight whether the *C. lectularius* microbiota can affect the immunity and vectorial capacity of its host. Thus far, the role only of *Wolbachia* in the immunity of *C. lectularius* has been investigated.

4.1 *Wolbachia*-induced immunity in *C. lectularius*

Wolbachia (order *Rickettsiales*) are Gram-negative, α -proteobacteria residing inside vacuoles of their hosts' cells [98]. *Wolbachia* symbionts cannot grow outside host cells but can be maintained in cell-free medium for short periods of time [99]. *Wolbachia* symbionts do not naturally occur in *A. aegypti* yellow fever mosquitoes which are major vectors of pathogens that cause debilitating and deadly diseases in humans (e.g., Dengue fever, Chikungunya, Zika [100, 101]. Experimental infections of *A. aegypti* with *Wolbachia* bacteria reduced virus loads and virus transmission rates [102, 103], and lowered loads of the protozoan parasite *Plasmodium falciparum* which causes malaria in humans [100, 104]. In combination, these data suggest that *Wolbachia* symbionts upregulate the expression of host immune genes, and increase host resistance to pathogens by priming host immunity [91]. The specific mechanisms by which *Wolbachia* symbionts enhance host immune responses are not known but may involve melanization (see above). Experimentally infected with *Wolbachia*, the vinegar flies *D. melanogaster* and *D. simulans* as well as *A. aegypti* had higher melanization rates, suggesting that melanization is one of the mechanisms by which *Wolbachia*

boost the immune response of their insect hosts [105]. In *A. aegypti*, *Wolbachia* symbionts upturned the production of AMPs, defensin and cecropin [102].

The essential nutrients riboflavin and biotin are absent from blood, and *C. lectularius* cannot produce them *de novo* [97]. To obtain these nutrients, *C. lectularius* relies on a mutualistic relationship with *Wolbachia* bacteria. In both field and laboratory settings, all instar nymphs and adult females and males of *C. lectularius* harbor *Wolbachia* symbionts in a specialized organ, the bacteriome, which is attached to the gonads [97]. *Wolbachia* symbionts are essential to the reproduction and survival of *C. lectularius*. In their absence, eliminated by antibiotic treatment, the reproductive fitness of adult *C. lectularius* was lower, and egg and nymphal development was hindered [106]. *Wolbachia* symbionts spread through body tissues and are transferred to offspring via maternal transmission [97].

There is also some degree of *Wolbachia*-mediated immunity in *C. lectularius*. With experimentally lowered *Wolbachia* loads, the survival of *C. lectularius* in response to microbial challenge decreased [64]. Heat-induced reduction of *Wolbachia* loads had no effect on LLA in the spermatheca and hemocoel and the volume of encapsulating cells, but it significantly decreased melanization responses in the spermatheca which – normally – mitigate wounding and infection costs associated with traumatic insemination [107]. Conversely, *Wolbachia* may not lessen the impact of viral infections in *C. lectularius*. The titer of the *Feline calicivirus* virus did not differ between *Wolbachia*-positive and *Wolbachia*-negative groups of *C. lectularius* [96]. The mechanisms by which *Wolbachia* symbionts confer at least some degree of immunity to their *C. lectularius* hosts warrant investigation.

5. Bactericidal activity of alarm pheromone components of *C. lectularius* and *C. hemipterus*

Under stress, nymphs, females and males of *C. lectularius* and of the tropical bed bug, *C. hemipterus*, all emit the alarm pheromone components (*E*)-2-hexenal and (*E*)-2-octenal from their metathoracic scent glands [108–110]. The ratio and amounts of these components differ between nymphs and adults of both species, and nymphs – unlike adults – also emit 4-oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal [109]. Emission of these compounds may not only signal distress but also contribute to the microbial defense of *C. lectularius* and *C. hemipterus*. Antimicrobial activity of these aldehydes has been demonstrated in two studies. (*E*)-2-Octenal suppressed growth of the Gram-positive bacterium *Staphylococcus albus*, but not of the Gram-negative *E. coli* [111]. Nymphs and adults of the pentatomid hairy shield bug, *Dolycoris baccarum*, emit a blend of (*E*)-2-hexenal, (*E*)-2-octenal and 4-oxo-(*E*)-2-hexenal, with each blend component expressing dose-dependent antibacterial activity against Gram-positive *Micrococcus luteus* and Gram-negative *Methylophilus* spp., *Pseudomonas* spp. and *Spirosoma* spp. [111].

Chronic infestations of *C. lectularius* affect the microbial community of household dust [95]. The microbiome of homes infested with *C. lectularius* differs from that of uninfested homes, but microbiome characteristics of uninfested homes return following eradication of *C. lectularius* [95]. Whether the antimicrobial activity of *Cimex* alarm pheromone components affects the microbial community of households, and the transmission and impact of bacteria to human hosts warrants investigation.

6. Conclusion

In this review, we summarize currently known immune responses by *C. lectularius* and propose worthy research topics. Challenged by microbe ingestion or infection, *C. lectularius* mounts cellular immune responses such as phagocytosis of bacteria and encapsulation of parasites, and humoral responses such as AMP secretion into the hemolymph. Infections also activate signaling pathways such as the Toll, IMD, and JAK–STAT pathways but the particular pathway that is activated by specific pathogens is still largely unknown. The functional immune system of *C. lectularius* resembles that of holometabolous insects but exhibits distinct deviations. They include a sparser immune repertoire, the production of DNA nets by hemocytes in response to pathogen invasions, and reproductive immune anticipation in the context of traumatic insemination. Many components of the *C. lectularius* immune system still await discovery. For example, the receptor molecules and immune pathways involved in antiparasitic and antiviral immune responses are yet to be discovered. Findings made in immune studies with holometabolic insects may serve as a guide to compare and confirm the functional role of candid immune molecules suggested by genome and transcriptome studies of *C. lectularius* [1, 5]. The definitive mechanisms that render *C. lectularius* incompetent vectors of disease-causing pathogens are hardly understood but seem to comprise – among others – upregulated LLA and AMP activity that help eliminate pathogens.

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Chapter 2

A Comprehensive Review of Non-Infectious Shell Disorders of Chelonia

Remco A. Nederlof, Doortje W. van Reekum, Job B.G. Stumpel and Jaco Bakker

Abstract

Chelonians are frequently presented to veterinarians with shell disorders of diverse etiology. This chapter provides a comprehensive review of non-infectious shell disorders in chelonians, emphasizing their pathophysiology, diagnosis, management, and prevention. Following an overview of relevant shell anatomy and development, detailed consideration is given to the triage and stabilization of traumatic and thermal shell injuries, including analgesia, antimicrobial therapy, wound debridement, and advanced wound management. Conventional and adjunctive modalities such as negative pressure wound therapy, laser therapy, and three-dimensional printing are discussed with respect to their efficacy and clinical applicability. Methods for shell fracture stabilization, including adhesive and hardware fixation techniques, are reviewed. Non-traumatic shell pathologies are explored with focus on neoplasia, congenital malformations, and husbandry-related conditions, including secondary hyperparathyroidism (metabolic bone disease), pyramidal growth syndrome, and shell ankylosis. For each disorder, diagnostic approaches, treatment principles, and welfare implications are outlined. Special emphasis is placed on preventive strategies addressing nutritional and environmental factors affecting shell health. By integrating clinical experience with existing literature, this chapter aims to provide clinicians with a practical and comprehensive guide for the management of non-infectious shell disorders in chelonians, promoting improved outcomes and welfare standards in both captive and wild populations.

Keywords: chelonians, turtles, tortoises, shell disorders, trauma, wound management, metabolic bone disease, pyramidal growth syndrome, ankylosis, husbandry

1. Introduction

The order Testudines, also known as Chelonia, within the class Reptilia includes all extant turtles, tortoises, and terrapins. It currently comprises 14 families, 97 genera, and approximately 352 species worldwide [1, 2]. Although vernacular usage varies regionally, in this chapter, tortoise refers to terrestrial species, terrapin to freshwater species, and turtle to marine species.

Both pet and wild chelonians are commonly presented to veterinarians for a range of medical conditions, among which shell disorders are particularly prevalent. A large UK study identified shell disorders as one of the most frequent health issues in pet chelonians, with reported prevalences of 9.7% in tortoises and 13.5% in terrapins [3]. In fact, shell disorders are the most frequent reason for presentation to a veterinarian in the UK after beak abnormalities and overgrown nails [3]. Traumatic shell injuries constitute a large proportion of shell disorders, particularly in tortoises, often resulting from vehicular collision, strimmer or lawnmower trauma, bite wounds from wild or domestic predator attacks, injuries from falls, or thermal injury [3–5].

Other commonly encountered shell disorders, such as pyramidal growth syndrome (PGS) and secondary hyperparathyroidism, colloquially referred to as metabolic bone disease (MBD), are often secondary to inappropriate husbandry [2]. Nevertheless, systemic and infectious disease may also predispose animals to shell disorders [3].

Infectious shell diseases of viral, bacterial, or mycotic origin represent an important subset of shell disorders. These infections can be primary, but may also be secondary to non-infectious shell disorders or underlying husbandry factors. Infectious shell disorders have been comprehensively reviewed elsewhere [6].

This chapter therefore focuses on the most frequently encountered non-infectious shell disorders of chelonians, i.e. thermal and traumatic shell injuries (subheader 4) and neoplastic, congenital, and husbandry-related shell disorders (subheader 5), outlining their clinical presentation, diagnostic approach, and treatment options, with emphasis on prevention through appropriate husbandry and management.

2. Methodology

To identify all relevant literature, we conducted a search for peer-reviewed publications, books, book chapters, newsletters, and conference abstracts across academic databases, including PubMed, EMBASE, CINAHL, POPLINE, Cochrane Library, Scopus, and Google Scholar. Publications primarily addressing shell disorders in which bacterial, viral, parasitic, or mycotic pathogens are considered to play a primary role were excluded, as these have been extensively reviewed elsewhere [6]. The retrieved literature was evaluated for relevance to non-infectious shell disorders, and sources were included or excluded based on their applicability to the research objectives. Following the identification of relevant non-infectious shell disorders, targeted literature searches were subsequently performed for each specific condition.

3. Anatomy of the shell

The development and anatomy of the chelonid shell are extensively described, and nomenclature for specific shell components may vary between sources [7–11].

The shell originates from a typical dermal-epidermal skin structure, in which the dermis becomes ossified to form bone, and the epidermis differentiates into a horny, keratinized covering. Development begins within the egg but continues post-hatching. Growth occurs by addition of new keratin layers beneath existing scutes, often producing visible growth rings, though these are unreliable indicators of age in captive chelonians [12].

The shell comprises two principal elements: the dorsal carapace and the ventral plastron, which are connected laterally by bony bridges, leaving cranial and caudal openings for the head and limbs.

The bony carapace consists of neural bones, fused with thoracic vertebrae, costal bones, which are fused with ribs, an anterior nuchal bone, and a lateral array of peripheral bones. The bony plastron consists in craniocaudal direction of the epiplastron, entoplastron, hyoplastron, hypoplastron, and xiphiplastron (**Figure 1**) [2].

Both carapace and plastron are externally covered by keratinized epidermal scutes, also termed scales or shields, arranged in a characteristic pattern. On the carapace, these include vertebral scutes, running along the midline, costal, or pleural scutes, which are lateral to the vertebral scutes, and marginal or peripheral scutes, lining the outer rim of the carapace. In some species, supra- or inframarginal scutes may be present dorsally or ventrally to the marginal scutes. The plastron is subdivided in craniocaudal order into gular, humeral, pectoral, abdominal, femoral, and anal scutes (**Figure 1**) [2]. The number and shape of scutes may vary among species. Adjacent scutes meet along epidermal furrows known as seams, which rarely coincide with the underlying bony sutures [12].

Soft-shell turtles (Trionychidae) lack horny scutes and are instead covered by a leathery skin. Although the center of the carapace still has bone underneath, the plastral bones are reduced, and peripheral, pygal, and suprapygal bones are absent [13]. The flexible shell conformation may facilitate visceral displacement during neck and limb retraction, facilitate gas exchange, and could be beneficial for swimming and burrowing into substrate [13].

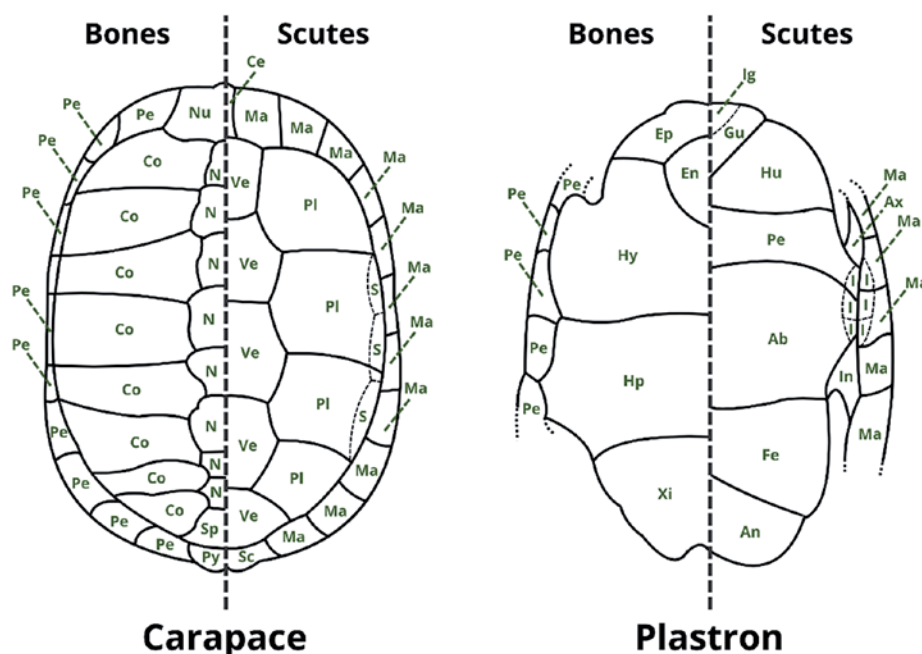


Figure 1. Schematic overview of the osseous and epidermal anatomy of the chelonid shell. Bones: Co, costals; En, entoplastron; Ep, epiplastron; Hp, hypoplastron; Hy, hyoplastron; N, neurals; Nu, nuchal; Pe, peripherals; Py, pygal; Sp, suprapygal; Xi, xiphiplastron. Scutes: Ab, abdominal; An, anal; Ax, axillary; Ce, cervical; Fe, femoral; Gu, gular; Hu, humeral; I, inframarginals; Ig, intergular; In, inguinal; Ma, marginals; Pe, pectoral; Pl, pleurals; S, supramarginals; Sc, supracaudal; Ve, vertebrals. © by R.A. Nederlof.

Plastral hinges have independently evolved in several genera and families of semi-aquatic (e.g. Kinosternidae, *Terrapene* sp., *Cuora* sp.) and terrestrial (e.g. *Kinixys* sp., *Pyxis* sp.) chelonians. These articulations allow varying degrees of closure of the head and limbs within the shell and may also assist in oviposition. Depending on the species, one or multiple hinges may occur anterior or posterior to the bridge, and in some cases, flexion may occur along the midline of the plastron [14, 15]. Hinges form through skeletal remodeling, wherein a bony gap develops and is occupied by connective tissue [14].

4. Thermal and traumatic shell injuries

4.1 Shell injury triage

The prognosis for shell fractures depends on the extent, location, and microbial colonization of the defect. An overview of the triage criteria is provided in **Table 1**. Cases of suspected spinal trauma should be confirmed using diagnostic imaging, particularly X-ray or computed tomography (CT), in combination with a full neurological assessment, including deep pain perception [2, 16]. Although chelonians have the necessary neuroanatomy to experience pain, it should be kept in mind that the response to a noxious stimulus may vary strongly between individuals and species, and deep pain perception may be difficult to evaluate [17].

4.2 Considerations for initial stabilization and hospitalization

When a chelonian presents with shell wounds or fractures, prompt stabilization and supportive therapy are essential. Analgesia should be provided throughout treatment; both opioids and NSAIDs are appropriate, depending on injury severity [18]. Antimicrobial therapy is indicated for all contaminated or infected wounds, ideally guided by bacterial culture and susceptibility testing [4]. The duration of antibiotic therapy can vary widely. Reported durations of antibiotic treatment ranged 13–216 days in one study, but should always be tailored to the individual patient [4].

Prognosis	Criteria
Excellent	Fresh (<6 h), non-displaced, closed, single fractures, hairline fractures, or fractures that do not involve the spine [2, 5].
Good	Multiple, unstable, or open fractures [5]. If large pieces of shell are absent, if the wound is contaminated or >24 h old, or if the coelomic membrane is damaged, the triage category should be downgraded [2].
Fair	Large shell portions missing, multiple fractures involving the shoulder or pelvic area, or penetrating punctures of the coelom [5]. If the animal is unable to move the leg, the prognosis for release is grave [2].
Guarded	Open fractures involving the coelom [5]. Extensive involvement of visceral organs may downgrade the triage category further [2].
Grave	Multiple comminuted fractures, especially if missing fragments exceed 30% of the shell surface or if the missing fragments are critical for stability. Grave fractures also include those coinciding with internal, head, or severe spinal injuries [5].

Table 1.
Triage criteria for traumatic shell injuries.

Because many individuals do not eat during hospitalization, placement of an esophagostomy tube may facilitate nutritional support and oral medication administration with minimal handling [5].

Dry docking is often necessary for terrapins and turtles during the initial stages of shell healing. Animals should be kept on a clean, non-abrasive substrate and provided with appropriate environmental humidity, an appropriate heat source, and access to UV-B light. Dry docking may be complete or partial, depending on the location and severity of the shell defect. Most terrapins can be kept out of water for 10–14 days; however, species with thinner, more permeable skin, i.e. musk turtles (*Sternotherus* spp.), are more susceptible to desiccation and therefore do not tolerate dry docking for extended periods of time. To minimize the duration of dry docking, gauzes impregnated with water-resistant ointment or watertight wound covers firmly adhered to the shell may be applied, so long as the extent and location of the wound permit [5, 19]. If a makeshift water-resistant wound cover is used, daily aeration is essential to prevent opportunistic bacterial and fungal infections [19]. Once a dry, mature granulation bed has formed, the wound can be safely exposed to water [5].

4.3 Thermal injury

Both burns and frostbite may affect the shell. It has been proposed that reptiles do not possess the same thermal withdrawal reflex observed in mammals and therefore may fail to recognize that a heat source is causing tissue damage [20]. However, an apparent lack of response to a noxious thermal stimulus may also indicate lethargy or an underlying systemic illness, highlighting the importance of a thorough clinical examination in all patients [21].

Burn injuries are most commonly caused by defective or improperly installed heating devices, such as under-floor heaters or overhead lamps positioned too close to the animal, though fires may also occur [2].

The keratinous scutes of the shell are relatively resistant to thermal damage and may initially appear unaffected. However, the underlying epidermal layers are often damaged, resulting in scute detachment days or weeks after the insult. If deeper tissues are involved, osteonecrosis of the underlying bone can occur (**Figure 2**). Scutes should not be forcibly removed until they begin to detach naturally, as they often remain firmly attached at the center. When scutes curl upward, only the detached margins should be trimmed to prevent the accumulation of devitalized tissue. Necrotic material can be removed once it separates from viable tissue. New bone plates will gradually form beneath the damaged areas, but healing may take several years, and the regenerated surface often differs in morphology from the original scute pattern [2].

Depending on the climate, frostbite may be observed in tortoises hibernated outdoors, particularly when unsuitable soils prevent adequate burrowing, resulting in hibernation too close to the surface [2]. Affected animals may frequently exhibit additional systemic signs such as pneumonia, rhinitis, or ocular abnormalities, which can worsen the prognosis. The gross appearance of frostbite lesions can resemble that of burns, although subscutal ecchymoses are typically present. These ecchymoses are usually self-limiting, and scutes should be left in place, even if mild exudation is observed. The prognosis is favorable if capillary perfusion can be restored [2].

For both burn and frostbite injuries, wound management is consistent with that of other shell injuries. Thermal wounds are particularly susceptible to secondary bacterial or fungal infections, and prophylactic antimicrobial therapy should be strongly considered [2].



Figure 2.
Carapace of a sulcata tortoise (Centrochelys sulcata) with burn wounds, showing differing stages of osteonecrosis and regeneration. © by D.W. van Reekum.

4.4 Advanced wound management

4.4.1 Traditional wound care

Once the patient is stabilized, wound management begins with thorough debridement under general anesthesia. Lavage with warm sterile saline or lactated Ringer's solution, or dilute antiseptic solutions such as povidone iodine (0.1–1%) or chlorhexidine (0.05–2%), removes debris from the wound [22, 23]. If the coelomic cavity is compromised, only sterile saline or lactated Ringer's should be used to avoid peritoneal irritation [2]. The animal should be positioned to allow the solution to drain from the wound site, and irrigation may need to be repeated over several days [2, 5].

Following initial lavage, the application of wet-to-dry dressings facilitates mechanical debridement and removal of necrotic tissue [2, 5, 23]. Dressings are changed daily, with debridement occurring at every bandage change. Silver-impregnated gauze or creams may be applied to reduce bacterial proliferation [5, 22, 23]. A variety of adjunct topical agents, e.g. granulated sugar, medicinal honey, wound gels, and borate-based glass products, can be used depending on wound type, healing stage, and treatment frequency [2, 4].

After approximately 1 month, a dry, leathery brown or gray granulation bed should form. From hereon, weekly monitoring is generally sufficient [5]. Complete healing of bony shell fractures is reported to require 6–30 months, depending on the extent and severity [5, 23, 24].

4.4.2 Adjunctive wound treatment modalities

Negative pressure wound therapy (NPWT), or vacuum-assisted closure (VAC), can be beneficial in managing complex or large shell wounds [25]. The benefits of NPWT include continuous approximation of soft tissue, removal of excess fluid and

exudate, increased blood flow, and direct stimulation of granulation tissue proliferation [26]. This results in reduced scarring and shorter healing times [27]. NPWT can be applied to most wounds, but should not be applied before aggressive debridement has been performed to prevent premature formation of granulation tissue over necrotic tissue [24, 27]. The process involves cutting open-cell polyurethane ether foam (pore size 400–600 μm) to fit the wound. Sensitive structures, such as exposed blood vessels or nerves, should be protected with non-adherent gauze. In addition, a silver-coated, non-adherent polyethylene mesh may be placed between the foam pad and the wound surface to reduce microbial growth underneath the foam pad [27]. The wound is sealed with an airtight film and connected to a vacuum port and suction tubing. A suction canister is included in the system to store suctioned moisture and exudate. A pressure control unit is used to set the negative pressure at approximately 125 mmHg (**Figure 3**) [24, 27]. Intermittent suction, e.g. 5 minutes on and 2 minutes off, may result in faster healing than continuous suction [28]. Intermittent suction, however, is reportedly less comfortable for the patient than continuous suction [27, 28]. It is advisable to start treatment of fresh wounds with continuous suction and to transition to intermittent suction after a while when inflammation has decreased [27, 28]. Dressings are changed every 1–3 days, and wound lavage is performed at each change. As the granulation bed matures, the frequency of changes may decrease [24, 27]. NPWT should be discontinued once a healthy granulation bed has formed, as ongoing use may slow epithelialization [29]. Although the necessity for NPWT materials seems cost-prohibitive, the reduced number of bandage changes, reduced time required for bandage changes, and reduced hospitalization times constitute economic advantages [27]. Moreover, cheaper VAC systems may be fabricated from spare suction tubing and aquarium pumps, but should be validated and equipped with appropriate pressure gauges and safety precautions [30].

Laser therapy, or photobiomodulation, is an economical, readily accessible adjunctive modality for wound healing in chelonians. It accelerates healing by inducing gene

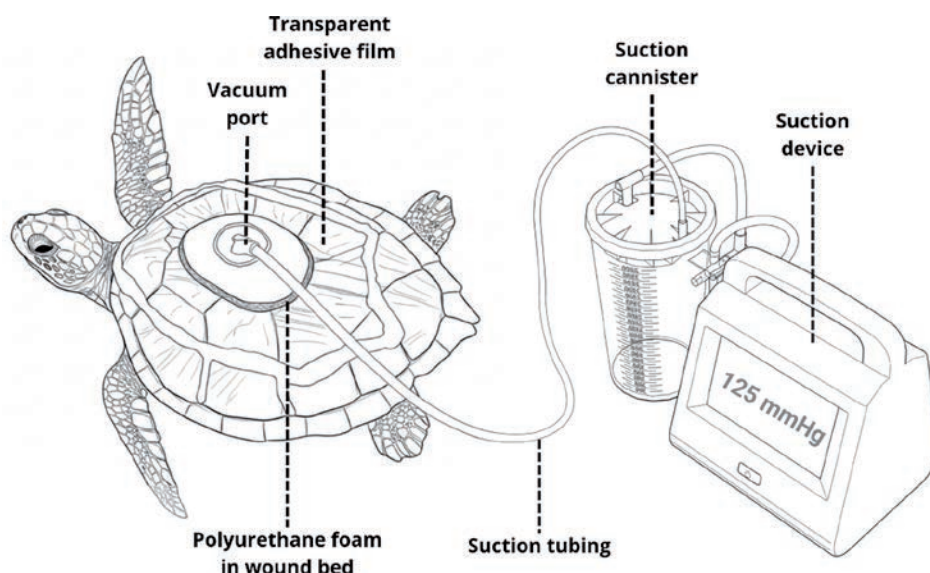


Figure 3. Overview of a basic NPWT setup for an aquatic chelonian. The flexible tubing and watertight seal may reduce the need for dry docking of (semi-)aquatic chelonians. © by R.A. Nederlof.

expression for growth factors, cytokines, and chemokines, increasing angiogenesis and cell proliferation, and acting as a decongestant by promoting vasodilation and lymphatic drainage [31]. In ulcerative shell lesions, repeated laser applications reportedly reduced lesion depth and erythema. Different wavelengths may be used, with shorter wavelengths (685 nm) suitable for superficial skin lesions and longer wavelengths (830 nm) for deeper lesions [32].

Three-dimensional printing is useful for large shell defects, particularly in aquatic chelonians, allowing early return to water and wound protection during keratinization. Designs should incorporate features for aeration and wound access to minimize the risk of infection and allow for wound treatment [33]. Limitations include the cost and availability of surface scanning and printing equipment, as well as the potential for pressure injuries if the model is ill-fitted. Although 3D printing may facilitate natural behaviors and protect the wound from contamination during recovery, no evidence currently supports accelerated healing rates with the use of 3D printing.

4.5 Shell fracture stabilization and repair

4.5.1 General principles and stabilization techniques

A variety of stabilization techniques have been described, including bandaging techniques, adhesive tapes, resins and composites, low-invasive hardware fixation (e.g. cable ties, hooks, clamps), and invasive hardware fixation (e.g. screws, wires, and plates). Stabilization techniques can be combined and changed throughout the healing process, depending on the fracture location and stability [2]. Because most shell fractures are older than 6 hours, they should be treated as contaminated or infected, and stabilization and wound therapy should precede surgical repair [22].

Generally, stable, non-displaced fractures can be managed with external fixation by means of bandages, adhesives, hooks, and ties. Hardware fixation with screws and plates is indicated for unstable and displaced fractures. Regardless of the repair technique, the surgeon should strive to adhere to standard aseptic techniques [4]. Moreover, fragment apposition should always be as close as possible, but care must be taken not to cause pressure necrosis. Eventually, both small and larger gaps will be undergrown by granulation tissue and ossifying coelomic membrane [2].

4.5.2 Adhesives

Sterile wounds, i.e. surgical wounds, may be repaired by direct application of glass ionomers, bone cement, photopolymerizable dental nanohybrid composite (PDC), or epoxy resins [2]. For traumatic injuries, direct application of adhesives is contraindicated, as they create an occlusive environment conducive to microbial growth. All adhesives should be removed after fracture healing, as they can cause shell distortion in growing animals [23].

Epoxy resins are the preferred method of closure for elective plastronotomies due to their ease of application. Their malleability and ability to adhere other elements to the shell constitute clear advantages. One must be careful, however, because the exothermic reaction during application may cause thermal trauma [5, 19, 34]. Epoxy has also been used to stabilize bone grafts, which may speed up the healing process [35].

Alternatively, ethyl cyanoacrylate activated with sodium bicarbonate, or simply put: super glue with baking soda, forms a strong, bactericidal, non-toxic compound. It cures faster than epoxy, permitting earlier tension application. Its flexibility of

application is equal to that of epoxy resins, but ethyl cyanoacrylate is often cheaper and more readily accessible. Moreover, it does not generate as much heat during curing, reducing the risk of thermal trauma [34]. For its application, the fracture site is manually apposed first. Then, ethyl cyanoacrylate is applied on the fracture edges and subsequently covered with sodium bicarbonate powder [34].

PDCs have been used to treat ulcerative shell defects. The process involves thorough debridement, application of EDTA solution to demineralize bone and help eliminate drilling residues, removal of residual moisture, and enamel etching to improve adherence. A bonding agent is applied and polymerized with a curing light in several layers, and the composite is then trimmed to the desired conformation [36]. In small ulcerations, healing is reported to occur within 1 month in 48% of cases, and all cases show healing and separation of PDC from healthy tissue within 90 days [36]. Disadvantages of PDCs are their availability and cost, as well as the need for a curing light.

An additional use of adhesives is to fix anchor points to the shell for low-invasive hardware fixation techniques [16].

4.5.3 Hardware fixation

Screw-and-wire fixation is the most common hardware fixation technique with reported success rates up to 71% in terrapins [4]. For this technique, pilot holes are drilled at least 0.5 cm from fracture margins, only partially penetrating the dermal bone [4]. A piece of tape may be placed around the drill bit to act as a depth guide [5]. Low drilling pressure and saline irrigation should be applied to prevent internal trauma and heat necrosis. Screws may also be advanced manually [4, 5]. Two screws are inserted on opposite sides of the fracture and joined with 20–22 gauge cerclage wire in a figure-of-eight configuration. The wire tips are twisted five to eight times around each other over the fracture site, bent flat, and covered with epoxy to prevent abrasion [2, 4, 5]. Metal plates may offer superior stability to cerclage wire, but are less adjustable during healing. Pilot holes should be predrilled into the bone and plates before fixation to the shell [4]. Used materials should preferably be made of surgical stainless steel. Galvanized screws should be avoided due to risks of zinc toxicity and rusting [2]. Aluminum or stainless steel plates and screws from a local hardware store constitute cheaper alternatives, but should be sterilized beforehand.

Alternatively, a cost-effective, low-invasive hardware fixation can be achieved using a combination of cable ties or metal wires, hooks, and other materials fixed to the shell. A TopClosure System®, consisting of two plates and an adjustable strap with a lock-release mechanism, has also been used successfully. The primary advantage is that the tension can be adjusted throughout the healing process without the need to fully undo the fixators [37]. Generally, however, fixation is less rigid than with screw-and-wire or metal plate fixation, which may prolong healing times and increase the risk of non-union fractures.

In very small chelonians, a combination of self-threading dental pins, Steri-strips, and adhesives may be used. Soft-shelled turtles are generally poor candidates for hardware fixation due to limited bone mineralization and resulting hardware instability [4].

Hardware removal may be staged according to fracture stability, and screw holes should be managed as minor shell wounds [4]. Reported complications include screw hole infection, fracture necrosis, hardware failure, and inadvertent penetration of the coelomic membrane with screws [4]. In a retrospective case-control study of 86 shell fractures, premature hardware removal was required in 26% of cases due to

failure to achieve fracture stability after 4 weeks. This was most often the result of fracture necrosis. Nevertheless, 80% of premature removals were still associated with a successful outcome [4]. Median healing time following fixation is 8 weeks, with a 65% success rate reported for open fractures involving the coelomic cavity [4]. Radiography is generally helpful in evaluating the healing progress, as ossified callous formation rarely occurs [16].

5. Non-traumatic shell disorders

5.1 Neoplasia

Neoplasia is considered to be relatively rare in chelonians, even among reptiles. In fact, neoplasia prevalence estimates in chelonians range from 0.34 to 1.2%, compared to 2.9–9% in squamates [38–40]. When neoplasia does occur, the skin is the most frequently affected site, particularly the limbs and mucocutaneous junctions, whereas the shell is less commonly involved [41]. As a result, most information on neoplastic disease of the chelonian shell is restricted to single-case reports.

A malignant melanophoroma, a type of chromatophoroma, originating from the caudolateral carapace was described in an 11-year old captive Hermann's tortoise (*Testudo hermanni*) [42]. The neoplasm presented as a slightly exudative, raised, compressible mass at the junction of the supracaudal scute and ventral edge of the fifth vertebral scute, causing elevation of the overlying scutes. The neoplasm infiltrated the underlying bone and could not be completely excised. Following surgical debulking, radiation therapy was initiated; however, the animal died 2 weeks later due to the effects of systemic metastases [42]. In other reptile species, surgical excision is reportedly curative in 30.3% of cases and is often difficult due to the infiltrative growth pattern of melanophoromas [43]. Radiation therapy may be used in conjunction, particularly in cases of incomplete excision, but success rates are low across reptile groups [43].

Basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs) affecting the carapace have been described in chelonians, including confirmed cases in Hermann's tortoises. Among 11 recorded SCCs in this species, only one originated from the epidermis of the shell [44]. It is important to note that certain stages of innate shell repair may exhibit hyperplastic and dysplastic epithelial folding, which can resemble SCCs both macroscopically and histologically [44, 45]. Invasive, osteolytic, non-metastatic, SCCs have been described in the carapace and plastron of Hermann's tortoises. The diagnosis should be based on biopsy and histopathological examination, complemented by CT imaging to assess the full extent of the lesion, which may be partially obscured by scutes or extend into deeper tissues. Surgical excision is the treatment of choice and is often curative [41]. In cases of incomplete removal, adjunct radiation or chemotherapy may be attempted, although these modalities are rarely effective as sole therapies [41, 46, 47]. However, as in other species, the prognosis is poor due to the invasive and metastatic nature of SCC [48].

5.2 Congenital malformations

Congenital malformations are caused by an interplay of genetic and environmental factors that disrupt the embryonic development [49–52]. It is thought that a disruption of signaling pathways during early morphogenesis interferes with the

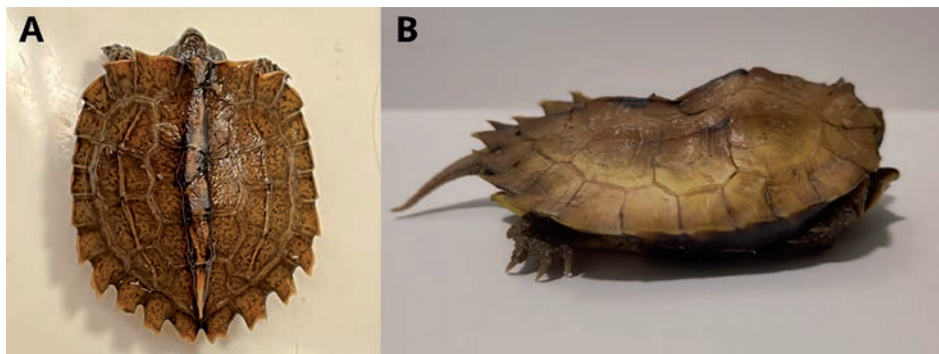


Figure 4. Congenital malformations in two juvenile Black-breasted leaf turtles (*Geoemyda spengleri*). (A) Top-down view demonstrating a congenital malformation of the vertebral scutes, supranumerary right costal scute, supranumerary left marginal scute, and a minor degree of scoliosis. (B) Lateral view demonstrating marked lordosis. © by J.B.G. Stumpel and D.W. van Reekum.

segmented organization of scute primordia, resulting in abnormal scute and plate formation, i.e. presence of additional plates and scutes, absence of regular shell plates, and unusual shape of plates and scutes (**Figure 4A**) [53, 54]. Congenital malformations may also include spinal deformities (see 5.3.3. Deformities and Ankylosis) (**Figure 4B**) [52]. Although the exact causes remain unknown, and are likely multiple, high environmental temperatures and low humidity during the incubation period are hypothesized to predispose animals to the development of congenital shell malformations [55, 56]. Although no conclusive correlation has been established, it is highly likely that environmental stressors and pollutants contribute to the increasing prevalence of congenital malformations in reptiles [52]. No therapy is required if the abnormalities do not limit the animal in growth, organ functions, or behavior. However, if the abnormality adversely affects the animal's health or welfare, treatment options should be evaluated. Depending on the type and severity of the defect, surgical correction may be appropriate if the prognosis is favorable. The feasibility and prognosis of surgical correction strongly depend on the specific nature and location of the malformation. Euthanasia should be considered in cases where the malformation is expected to compromise welfare despite therapeutic efforts.

5.3 Husbandry-related shell disorders

5.3.1 Secondary hyperparathyroidism (NSHP, RSHP, MBD)

Nutritional (NSHP) and renal (RSHP) secondary hyperparathyroidism, colloquially referred to as metabolic bone disease (MBD), are metabolic conditions arising from an interplay of environmental and physiological factors on the calcium and vitamin D metabolism of tortoises. The syndrome has been well-researched, and information on its exact causes, pathology, clinical signs, and treatment can be readily found in other literature [2]. In brief, when ionized calcium levels in the blood decrease, the parathyroid glands secrete parathyroid hormone (PTH). PTH stimulates intestinal calcium absorption, renal calcium reabsorption, and bone resorption, including mobilization of calcium from the shell. If dietary calcium intake or vitamin D availability is not corrected, this process persists, leading to progressive bone decalcification and shell softening. The condition may result from insufficient



Figure 5.

A severe case of NSHP and pyramidal shell growth in a red-footed tortoise (Chelonoidis carbonarius) secondary to inappropriate husbandry. © by D.W. van Reekum.

UVB exposure, inadequate cholecalciferol levels, calcium-deficient diets, imbalanced dietary calcium-to-phosphorus ratios, or chronic hepatic and/or renal disease [2].

Clinical signs commonly include shell softening, distortion of the carapace and plastron, collapse of the caudal carapace with associated lordosis resulting in hindlimb paresis, cloacal prolapse, inability to lift the shell off the ground when walking, dystocia, and pathological fractures (**Figure 5**) [57, 58].

The diagnosis is based on a thorough clinical history in combination with diagnostic imaging. Although X-ray is often used for initial assessment, CT can provide osteodensitometric measurements, facilitating earlier detection and more accurate assessment of the degree of bone demineralization [59].

The treatment aims to restore and maintain ionized calcium concentrations in both the short and long term. Supportive therapy and calcium administration can be utilized short term, but definitive treatment requires addressing identified underlying husbandry issues. Pathological fractures should be treated as other fractures, though caution is warranted due to the fragility of demineralized bone. Although deformities may be stabilized with appropriate treatment, complete restoration of normal anatomical conformation is rarely achievable. Consequently, severe deformities or fractures that compromise welfare by preventing the animal from expressing natural behaviors may justify euthanasia. Emerging therapeutic innovations include a mechanical bracing system designed to lift up the carapace into a more natural shape. This system may promote vertebral realignment, reinstate neural connections, and restoration of blood flow, potentially improving paresis posterior associated with advanced secondary hyperparathyroidism [2, 60]. Despite these advances, prevention and early intervention remain paramount, as welfare is invariably compromised once the disorder progresses. Even when secondary hyperparathyroidism is successfully stabilized, the cytotoxic effects of elevated parathyroid hormone levels predispose affected animals to renal disease later in life [61].

5.3.2 Pyramidal growth syndrome (PGS)

Pyramidal growth syndrome (PGS), or simply pyramiding, is a husbandry-related growth abnormality observed in captive tortoises. The condition is characterized by thickening of the trabecular bone between the bone cortices of the shell, producing

bony, pyramid-shaped protuberances on carapace. These protuberances remain covered by a normal layer of scutes (**Figures 5 and 6**) [62, 63].

Because PGS is only reported in captive animals, the cause has long been suspected to be related to improper husbandry, i.e. diet or housing conditions. It is now widely accepted that low environmental humidity is the primary causative factor of PGS [56, 62, 63]. Excess nocturnal heating, even under adequate humidity conditions, has also been implicated as an independent contributing factor to PGS development [64].

Although high-protein diets have been associated with PGS, this relationship likely reflects protein-related accelerated growth rates rather than a direct effect on shell formation [62]. Other proposed dietary contributors, i.e. low calcium intake and decreased alimentary calcium-to-phosphorus ratios, may also play a role; however, in the present literature, these factors often co-occur with low humidity, making causation difficult to isolate [57].

The pathogenesis is thought to involve dehydration, resulting from low environmental humidity, of the keratin-producing cells situated between the existing keratin scutes. Dehydration causes these growing cells to shrink, resulting in a depression between the existing keratin scutes. Subsequent growth exerts upward pressure on the surrounding keratin plates, which are elevated compared to the keratin-producing cells. This upward pressure results in the formation of pyramidal shell growths (**Figure 7**) [63].

While the process is irreversible, progression can be halted through the restoration of appropriate humidity levels, allowing the resumption of normal physiological growth patterns [63]. Preventive medicine by addressing inappropriate housing conditions is therefore paramount. Methods to maintain adequate humidity include the use of misters, foggers, and humid hides containing moist substrate. It is also critical to ensure that ambient and basking temperatures from heat lamps do not cause excessive shell dehydration, particularly on the very top of the shell. Most tortoises with PGS are able to lead normal lives, provided that the condition does not impair mobility, cause discomfort, or limit the expression of natural behaviors.

5.3.3 Deformities and ankylosis

Spinal deformities, and concurrent shell deformities, can have a range of underlying causes. Though frequently mentioned, physical trauma resulting in vertebral



Figure 6.
Two captive-raised adult male Egyptian tortoises (Testudo kleinmanni) of equal weight. The male on the left shows physiological carapace growth, the one on the right shows advanced pyramidal growth. © by J.B.G. Stumpel.

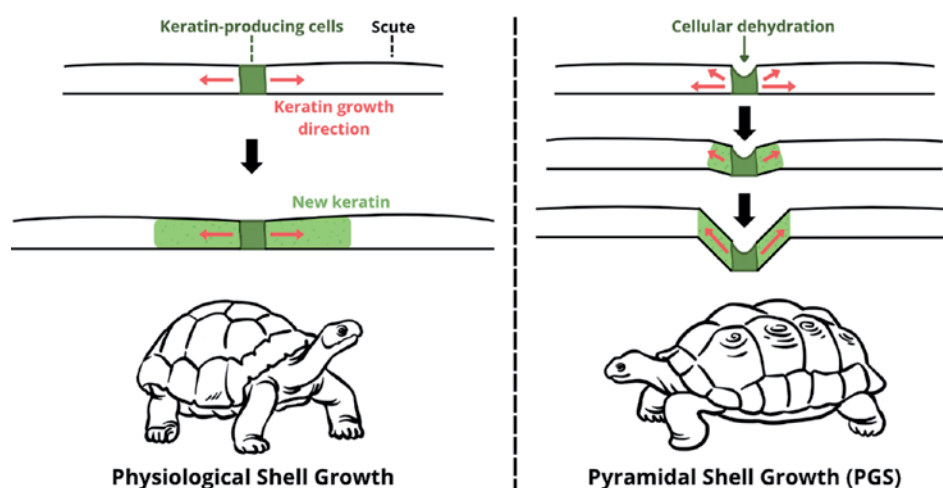


Figure 7. Schematic illustration of the (patho)physiology of normal versus pyramidal shell formation in tortoises. © by J.B.G. Stumpel and R.A. Nederlof.

fractures is likely to be only a sporadic cause of spinal deformity [65]. Pathological fractures resulting from metabolic bone diseases are more commonly observed in captive chelonians, whereas factors previously described to contribute to congenital abnormalities are the leading cause in wild animals [52].

The most frequently observed types of spinal malformations in chelonians are kyphosis, scoliosis, lordosis, and kyphoscoliosis. Kyphosis is reported to occur significantly more frequently in (semi)aquatic species compared to terrestrial species [52]. Kyphotic turtles have been reported from many chelonian groups with estimated wild incidences of 0.08% in marine turtles and 0.10–0.36% in terrapins [66–68]. Most kyphotic turtles appear to be able to feed and reproduce readily, thus showing this deformity may have minimal affects on a turtle's biology.

Ankylosis of the shell refers to the premature or partial fusion of shell sutures, resulting in restricted growth and deformation of the carapace [69]. The condition may occur secondary to trauma or inadequate environmental or nutritional factors, similar to those reported to predispose for NSHP.

Intervention is warranted if the deformity affects normal organ function or behavior of the animal. Depending on the severity and associated complications, surgical correction may be indicated. Procedures such as shell-widening or ovariectomy may be performed in cases of obstructive dystocia resulting from shell deformity [70]. However, if surgical resolution is not feasible or expected to improve long-term welfare to acceptable standards, euthanasia should be strongly considered.

6. Conclusions and considerations

Non-infectious shell disorders in chelonians are frequently encountered in veterinary practice and are most often traumatic or husbandry-related in origin. As nearly all such disorders are linked to inadequate environmental or nutritional management, effective treatment requires not only evidence-based medical intervention but also a thorough assessment and correction of husbandry practices. Particular attention

should be given to optimize the animal's environment and diet. Although detailed species-specific husbandry guidelines are beyond the scope of this chapter, it is essential that clinicians possess a thorough understanding of species-specific behaviors, environmental needs, and nutritional requirements to ensure effective treatment and, more importantly, prevention of most non-infectious shell disorders.

Future research should focus on refining and advancing treatment modalities for the range of non-infectious shell disorders. For shell injuries, more widespread application of NPWT and further research into adjunct healing modalities, such as laser therapy and 3D printing, are required to reduce healing times and improve patient comfort and treatment success. Although the precise etiologies of some shell disorders remain unclear, this uncertainty must not hinder the application of existing evidence-based preventive measures focused on optimal husbandry and welfare. Concurrently, research should focus on improving our understanding of the mechanisms underlying the development of neoplastic, congenital, and metabolic shell disorders, and improve prevention and management.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

BCC	basal cell carcinoma
CT	computed tomography
EDTA	ethylenediaminetetraacetic acid
MBD	metabolic bone disease
NPWT	negative pressure wound therapy
NSAID	non-steroidal anti-inflammatory drugs
NSHP	nutritional secondary hyperparathyroidism
PDC	photopolymerizable dental nanohybrid composite
PGS	pyramidal growth syndrome
PTH	parathyroid hormone
RSHP	renal secondary hyperparathyroidism
SCC	squamous cell carcinoma
UV-B	ultraviolet B
VAC	vacuum-assisted closure

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Section 2

Animal Nutrition

The Role of Phytogetic Feed Additives in Modulating Poultry Nutritional Physiology and Genomics

Hasan Hüseyin Ipçak

Abstract

In this chapter, the effects of phytogetic feed additives (PFAs) on the performance, intestinal health, microbiota, oxidative stress, antioxidant defense system, gene expression, and protein production of poultry were examined. PFAs consist of plant-derived components such as essential oils, flavonoids, saponins, phenolic compounds, and alkaloids. They have gained increasing popularity in recent years as alternative growth-promoting products to antibiotics used for animal nutrition. Studies have indicated that PFAs positively affect the overall health and productivity of poultry. Indeed, it has been observed that the use of PFAs in poultry nutrition enhances performance, modifies intestinal microbiota and morphology, and modulates gene expression and protein synthesis. Thus, the use of PFAs in poultry farming is an important alternative to increasing productivity and transitioning to a more sustainable and natural production system. However, further research is needed to understand the efficacy and safety of PFAs, interactions between different phytogetic components, and their synergistic effects. Furthermore, determining the appropriate levels and application methods is crucial to guide producers and reach a wider audience in poultry farming. In conclusion, supporting the use of phytogetic feed additives within the framework of legal regulations and standards is important for the sustainability of the industry and health of consumers.

Keywords: poultry, phytogetic feed additives, nutritional physiology, genomics, intestinal health

1. Introduction

Poultry farming has persistently grown into an intensive livestock sector worldwide, despite increasing poultry product demand and emerging pathogen threats, thanks to advancements in areas such as feeding, disease control, genetic improvement, and dietary requirement management [1]. Therefore, this sector requires sustainable, therapeutic, and production-supporting additives, especially those based on phytogenics, which offer advantages such as low cost, easy availability, residue-free

nature, and nonantibiotic resistance threats [2]. Phytogenics, also known as phyto-biotics, are natural bioactive compounds of botanical origin that are used as alternatives to antibiotic growth promoters (AGPs) and are added to feed to enhance animal performance [3, 4]. Although phytogenics encompass a wide variety of substances of biological origin, formulation, chemical characterization, and purity, they can be classified into four groups [5]: (1) herbs (products obtained from flowering, woody, and nonperennial plants); (2) botanicals (whole or processed parts of a plant, such as roots, leaves, tree bark, and spices); (3) essential oils (hydro-distilled extracts of volatile plant compounds); and (4) oleoresins (anhydrous solvent-based extracts). These plant-derived products do not contain residues, are natural, and are less toxic than synthetic antibiotics or inorganic chemicals [6]. Many are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA) and can be used as feed additives in animal nutrition [7, 8]. This class of feed additives has recently garnered increasing interest, particularly in poultry farming. One of the main reasons for this increased interest is the significant increase in the number of scientific publications in this area since the 2000s [4].

Numerous studies have reported various functions of phytogenic feed additives (PFAs), including antimicrobial, antiviral, antioxidative, antihelminthic, anti-inflammatory, anti-stress, and nutrigenomic effects, as well as improvements in feed palatability, intestinal development, and health [9, 10]. In addition, they have been reported to stimulate growth, modulate immunity, promote feed intake and endogenous enzyme secretion, and increase production [8]. The beneficial effects of PFAs are generally attributed to their antimicrobial and antioxidant properties [11]. However, some proposed theories for the mechanisms underlying the functions of PFAs, despite their large-scale uncertainty, include: (1) Inhibition of pathogens (reducing infection) and reduction of the total bacterial load in the gut (providing more energy and nutrients for animals) through strong antimicrobial effects, which damage pathogenic bacterial cells' glycolipid walls [3, 10, 12]; (2) Improving animal health by reducing oxidative stress and increasing antioxidant activity in various tissues [13, 14]; (3) Enhancing the activity of lymphocytes, macrophages, and natural killer (NK) cells, increasing phagocytosis, or stimulating interferon synthesis through immunostimulatory effects [6, 15]; (4) Activation of toll-like receptors, luminal capture by dendritic cells, stimulation of epithelial cells, and secretion of proinflammatory cytokines in the mucosa [16]; (5) Expansion of the absorptive surface of the intestine through elongation of villi and deepening of intestinal crypts [3]; (6) Improving nutrient digestibility by increasing intestinal and pancreatic enzyme production and activity, as well as bile flow [17]; (7) Stabilizing the microbiome by reducing microbial toxins (e.g., ammonia and biogenic amines) [4]; (8) Supporting the proliferation of beneficial bacteria such as *Lactobacilli* and *Bifidobacteria* [6] are among the suggested mechanisms for PFAs.

The biological and therapeutic activities of PFAs are closely related to the plant chemicals (primary and secondary compounds) present in the structure of phytogenics [9]. Primary compounds are considered the main nutrients (protein, fat, and carbohydrates), while secondary compounds include various bioactive components, such as alkaloids (alcohols, aldehydes, ketones, esters, ethers, and lactones), bitters, flavonoids, glycosides, mucilage, saponins, tannins, phenolics, polyphenols, terpenoids (mono- and sesquiterpenes, steroids), polypeptides, thymol, cineole, linalool, anethole, allicin, capsaicin, allyl isothiocyanate, and piperine [3, 6, 8]. Numerous variations exist in the bioactive composition of PFAs owing to biological factors (plant species, parts, age, growth location, and harvest conditions), processing

techniques (extraction, distillation, and stabilization), storage conditions (light, temperature, oxygen tension, and time), climate, and geographical conditions [18]. Consequently, the expected effects of plant extracts and plant-derived products also vary. Therefore, it is critically important to individually examine the specific effects of phytogetic compounds on animal physiology and genomics to facilitate their dietary application [10]. This section focuses on research conducted to better understand the effects of phytogetic feed additives on the performance and health of poultry. In line with this objective, this section discusses in detail the mechanisms of action of these additives on factors such as gut health, immune system, antioxidant defense system, and gene and protein expression.

1.1 Effects of phytogetic feed additives on performance

PFAs, including plant extracts, essential oils, bioactive compounds, and oleoresins, can be used as growth promoters in poultry nutrition because of their versatile biological activities. When PFAs are incorporated into poultry diets, they influence the metabolism by combating stress and microbial activity [2]. Numerous studies have reported positive effects of PFAs on broiler chicken body weight gain (BWG) [17, 19–25]. Various strategies have been proposed to understand this positive effect on poultry BWG. Windisch et al. [4] reported that PFAs positively affect the gastrointestinal microbiota ecosystem and gut eubiosis by stabilizing feed hygiene and controlling potential pathogens, based on reviews by Eckel et al. [26] and Roth and Kirchgessner [27]. Consequently, owing to more stable gut health, animals are less exposed to microbial toxins, ammonia, other undesirable microbial metabolites, and nutrient competition. Thus, PFAs help animals gain more body weight by strengthening the host immune system and increasing the gut availability necessary for nutrient absorption. The effects of PFAs on growth performance of broiler chickens and Japanese quail are presented in **Table 1**.

Platel and Srinivasan [37] attributed the improvement in gut function primarily to the potential stimulatory effect of PFAs on digestive secretions such as digestive enzymes, bile, and mucus. Similarly, Khan et al. [38] associated improved performance with increased secretion of digestive enzymes such as lipase, amylase, trypsin, and chymotrypsin, leading to greater nutrient utilization in the liver. Furthermore, essential oils can specifically enhance protein digestion by increasing hydrochloric acid and pepsin secretion [39] and improve fat digestibility by modulating bile salt, pancreatic, and intestinal lipase production [40]. The stimulatory effects of essential oils on feed intake may result from increased palatability due to enhanced taste and aroma or from the suppression of unpleasant flavors in other feed ingredients [15]. However, since broilers are not sensitive to smell, this effect may stem from the appetite-stimulating properties of feed that trigger the secretion of saliva and gastric juices. As a result, they may improve their BWG due to increased feed consumption [41].

The gastrointestinal system is the main region for nutrient absorption and the primary site of the chemosensory system, which contains numerous nerves and receptors [42]. Recent studies have shown that receptors such as taste, calcium-sensing, and lipid receptors are prominent in the intestine [43]. The intestinal epithelium comprises approximately 90% absorptive epithelial and enteroendocrine cells with nutrient-carrier proteins. Nutrient carriers are located in both the brush border membrane for the transport of nutrients from the gut lumen into the cell, and the basolateral membrane for the transport of nutrients into or out of the bloodstream [44].

Animals	PFAs	Inclusion rate	Treatment effect (% difference from untreated control)				Reference
			Final BW	BWG	FI	FCR	
1-day-old Ross308 broilers	Mixture of Capsicum oleoresin, carvacrol, cinnamaldehyde	150 mg/kg	↑ 4.63	↑ 4.31	↑ 2.77	↓ 4.56	[28]
1-day-old Cobb 500 broilers	An EO blend from star anise, rosemary, thyme, and oregano EO	25 mg/kg	↑ 5.59	↑ 3.57	→	↓ 2.24	[29]
1-day-old Arbor Acre broilers	Commercial EO (3.05% thymol, 2.3% carvacrol, and 0.26% cinnamaldehyde, carrier is dextrin)	50, 100, 200 and 400 mg/kg	→	→	→	→	[30]
1-day-old male yellow feather broilers	Wisdem EO	400 mg/kg	↑ 5.01	↑ 3.98	→	↓ 4.55	[31]
1-day-old Ross308 broilers	Mixture of mint, parsley, and carrot seed oils (EOs)	1.5 g/kg	↑ 5.14	↑ 5.24	→	↓ 5.11	[32]
	EOs + black pepper oil	1.5 + 0.5 g/kg	↑ 7.22	↑ 7.31	→	↓ 8.52	
	EOs + radish seed oil	1.5 + 0.5 g/kg	↑ 9.16	↑ 9.30	→	↓ 9.09	
Three day old Ross308 broilers	Thyme EO	150–300 mg/kg	→	→	→	→	[33]
	Rosemary EO	100–200 mg/kg	→	→	→	→	
1-day-old Cobb 500 broilers	<i>Emblica officinalis</i> (Amla) extracts	3 g/kg	↑ 0.82	↑ 0.77	↓ 1.80	↓ 2.42	[34]
	<i>Cichorium intybus</i> (Chicory) extracts	3 g/kg	↑ 2.68	↑ 2.72	↓ 1.84	↓ 4.24	
	<i>Allium porrum</i> (Leek) extracts	3 g/kg	→	→	↓ 1.18	↓ 1.21	
7-day-old Japanese Quails	Aloevera leaf powder (AV)	0.5%		↑ 4.76	↓ 3.57	↑ 6.12	[35]
	Clove powder (CV)	0.5%		↑ 4.00	↓ 4.15	↓ 7.55	
	AV + CV	0.25 + 0.25%		↑ 6.82	↓ 4.64	↓ 10.43	
Japanese Quails initial BW 9.15 ± 0.12)	Black cumin powder	1, 2, and 4%		→	→	→	[36]

PFAs, phytogenic feed additives; EO, essential oil; BW, body weight; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio; ↑, increase; ↓ decrease, → no significant differences.

Table 1.
The effects of PFAs on growth performance in broiler chickens and Japanese quails.

Hundal and Taylor [45] reported that nutrient carriers also contributed to the detection of luminal nutrients as receptors. Therefore, nutrients, such as amino acids, peptides, sugars, fatty acids, minerals, and vitamins, can be detected by intestinal chemosensors [10]. Indeed, it has been reported that PFAs can regulate the gene expression profile of ileal mucosa [46, 47]. In this context, PFAs can increase nutrient absorption by increasing the expression of nutrient carrier genes, thereby enhancing performance efficiency. In this context, previous studies showing the effects of PFAs on laying performance in hens and Japanese quails are presented in **Table 2**.

1.2 Effects of phytogetic feed additives on the immune system

Immunomodulation is the manipulation of the immune system by pharmacological means [55]. Nutritional immunomodulation can be defined as the use of specific dietary nutrients to achieve a targeted goal and to alter aspects of immune function. Many nutrients modulate the immune system. The immune system in poultry is highly complex and multifactorial. Therefore, to fully benefit from nutritional immunomodulation, it is necessary to understand how the immune system of poultry functions [56]. The primary function of the immune system is to recognize foreign substances entering the body and to initiate and manage appropriate physiological responses to neutralize or eliminate them [57]. The immune system of poultry has two main components: innate and acquired immunity [56]. Innate immunity is the first nonantigen-specific defense mechanism against pathogenic microorganisms. Leukocytes such as macrophages release cytokines in response to pathogen invasion, which enables pathogen recognition, initiates the immune response, and leads to inflammatory reactions [58]. Poultry has barriers, such as the skin and mucosal membranes, that protect the body against pathogens. If pathogens pass through this epithelial barrier, they are recognized, internalized (phagocytosis), and broken down by cells of the innate immune system (neutrophils and macrophages) through Toll-like receptors (TLRs) and pattern recognition receptors [59].

If the innate immune system is unable to eliminate pathogens and the number of pathogens reaches an antigen threshold, the acquired immune response is activated [60]. Acquired immunity is a specific immune system initiated by the emergence of antigens. Pathogens and exogenous stimuli are identified and eliminated by antibody-producing B cells and cytotoxic T cells that actively kill specific invading pathogens [56, 61]. In addition, these cells secrete a range of communication molecules, such as lymphokines and cytokines (interleukins and interferons), when they encounter pathogens. These factors subsequently detail the immune response by directing cells toward a specific pathogen [62]. Two main pathways modulate the immune response. One is the activated protein kinase mitogen-activated protein kinase (MAPK), which mediates the regulation of gene expression [63], increases intracellular communication and extracellular stimulation [64], and ultimately forms an appropriate biochemical and physiological cellular response. Another factor is the nuclear factor kappa B (NF- κ B). NF- κ B plays a crucial role as a transcription factor in inflammatory processes. Upon activation by various inducers, including reactive oxygen species (ROS) and bacterial lipopolysaccharides (LPS), NF- κ B translocates from the cytoplasm to the nucleus and synthesizes a wide range of proinflammatory proteins, such as cytokines, chemokines, adhesion molecules, and enzymes involved in inflammation, apoptosis, and cell proliferation [10]. There are various interactions between the MAPK and NF- κ B signaling pathways and PFAs, particularly among essential oils.

Treatment effect (% difference from untreated control)							
Animals	PFAs	Inclusion rate	Egg number	Egg pro.	Egg weight	FCR	Reference
32 weeks old Lohmann Brown Lite hens	Fennel seeds	0.5%	↑11.29	↑11.32	↑3.46	↓19.02	[48]
	Black cumin seeds	0.5%	↑4.89	↑4.89	↑0.22	↓9.51	
	Hot red pepper	0.5%	↑7.47	↑7.49	↑1.26	↓13.44	
30 weeks old Lohmann LSL- Lite hens	Rosemary EO	20 ml/100 kg		→	→	→	[49]
	Dill EO	15 ml/100 kg		→	→	→	
	Chicory extract	250 ml/100 kg		→	→	→	
54 weeks old Lohmann Brown hens	Tea tree <i>Melaleuca alternifolia</i> (Maiden and Bette) Cheel EO	80 mg/kg		↑4.24	→	→	[50]
42 weeks old Lohmann LSL- Lite hens	Lavender EO (LEO)	250 mg/kg		↑3.13	→	→	[51]
	Mint EO (MEO)	250 mg/kg		↓5.35	→	↑9.73	
	LEO + MEO	250 + 250 mg/kg		↓5.61	→	↑6.19	
10 weeks old Japanese quails	Chamomile extract, wild mint extract, or oregano extract.	1 mL/L drinking water		→	→	→	[52]
7 weeks old Japanese quails	<i>Moringa oleifera</i> seed powder	0.1%		↑21.89	↑7.10	↓9.65	[53]
		0.2%		↑18.89	↑11.55	↓19.94	
		0.3%		↑34.48	↑9.05	↓20.58	
10 weeks old Japanese quails	Ginger root powder	0.5, 1.0, or 1.5 g/kg		→	→	↓10.23	[54]

PFAs, phytogetic feed additives; EO, essential oil; Egg pro., egg production; FCR, feed conversion ratio; ↑, increase; ↓ decrease, → no significant differences.

Table 2.
The effects of PFAs on laying performance in hens and Japanese quails.

ROS, LPS, mitogens, or toxins produced under stress conditions can cause significant damage to intestinal cells. They can subsequently induce inflammation and immune responses by activating NF- κ B signaling, leading to increased expression of proinflammatory cytokines, such as interleukin-1 (IL-1), IL-6, IL-8, IL-15, IL-18, interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α), and chemokines. This can result in reduced production efficiency. Indeed, it has been reported that broilers infected with *Eimeria* spp. and fed a diet supplemented with 14.4 mg/kg cinnamaldehyde showed increased mRNA expression of proinflammatory cytokines IL-1 β , IL-6, IL-15, and IFN- γ [65]. Cytokines are a group of nonantibody proteins or peptides that serve as mediators between cells and facilitate cell communication [66]. They are typically released when needed, particularly during infections and inflammation, and are not stored. Cytokines are involved in both innate and acquired immune responses and signal immune system cells, such as T cells or macrophages, in the presence of pathogens to migrate to the site of infection. Their intercellular signaling can be variable, sometimes leading to protective or damaging responses [66]. However, reduced exposure to pathogenic bacteria or their toxic metabolites due to the antimicrobial effects of PFAs, particularly essential oils, may result in less need for immune defense activity in the gut [67]. Similarly, the antimicrobial effects of FYKs may reduce the activation of inflammatory responses and, consequently, the growth-suppressing effects associated with them [56]. It has been found that adding a mixture of citrus peel, thyme, and anise essential oils (40 mg/kg) to the diets of piglets reduces NF- κ B and TNF- α gene expression, resulting in an anti-inflammatory effect [68]. In another in vitro study, it was reported that cinnamaldehyde, the main bioactive component of cinnamon, and Cinnamomum cassia oil inhibited mRNA expression of proinflammatory cytokines, including IL-1 β , IL-6, and TNF- α , in LPS-activated J774A.1, while increasing the expression of anti-inflammatory cytokine IL-10 [69].

There is a balance between the reciprocal responses of the innate and acquired immune systems [61]. In monogastric animals, innate immune responses are required when encountering intracellular pathogens such as *Escherichia coli* or *Salmonella* spp., whereas acquired immune responses are necessary to combat extracellular pathogens such as *Staphylococcus aureus*, *Listeria monocytogenes*, and helminths [70]. Therefore, enhancing the function of the acquired immune system can make animals susceptible to pathogens that are best recognized and eliminated by the innate immune system, and vice versa [56]. These two systems must be constantly kept balanced.

1.3 Effects of phytogetic feed additives on intestinal health and microbiota

Modern poultry production practices have undergone dramatic changes over the past 50 years. The high growth rates and reproductive characteristics generated through genetic selection, along with improved management techniques and meeting nutritional requirements, have resulted in increased performance standards across all animal farms. However, it is still unclear whether animal performance will reach a plateau due to genetic and/or physiological limitations in the near future. Alongside these limitations, the term “gut health” has become the focus of the poultry industry and scientific research in the last decade [71, 72]. Today, there has been an exponential increase in the use of terms such as “gut health,” “intestinal health,” “enteric health,” or similar terms in scientific literature, and great interest has been shown in defining this concept. Despite the lack of a clear definition of gut health, scientific data are insufficient to fully explain what gut health is, how it can be defined, and, most importantly, how it can be measured. Gut health is a prerequisite for optimum

performance and profitability in poultry production [41]. The intestine exhibits various functions, such as absorption of nutrients, electrolytes, and water, secretion of mucins and immunoglobulins, and selective barrier protection against harmful antigens and pathogens [10, 73]. Furthermore, in recent years, they have been shown to play an active role in organ (intestinal) integrity and body defense [74]. The mucosal layer of the intestine, which has a large absorption surface area, can be damaged by factors such as viruses, bacteria, fungi, toxins, or ROS, leading to impaired gut health. When inflammation occurs, the body's reaction takes place in the form of accelerated renewal of damaged tissues and secretion of proinflammatory cytokines and chemokines [61]. This process is complex and requires additional energy to gather immune cells that are distant from the site of infection. Consequently, the body increases its metabolic rate (fever) and reduces its feed intake [56]. Therefore, by alleviating or preventing excessive inflammation, gut health can be preserved, thereby providing benefits to poultry production. With reduced inflammation, protein production can be allocated to growth rather than to immune modulators [75]. Thus, the use of PFAs in poultry nutrition can help prevent gut disorders. It has been reported that PFAs can stabilize gut health by reducing microbial pressure [4, 76].

Bacterial diseases continue to pose a significant problem to poultry production [77]. Microbial diseases such as *Clostridium perfringens*, *E. coli*, *Salmonella* spp., *S. aureus*, and *Eimeria* spp. have been causing concerns for many years. Indeed, in a feeding program without the use of AGPs, poultry rely heavily on their immune systems to prevent bacterial infections [56]. However, under intensive farming conditions, where more animals are in close contact and exposed to various disease-causing agents, relying solely on their immune systems is insufficient to combat these factors. This can lead to increased productivity losses and mortality rates. Notably, the removal of AGPs from broiler diets in the European Union has led to a significant increase in the incidence of necrotic enteritis (NE). Although the exact effect of AGPs remains undetermined, their withdrawal from the animal feed is directly associated with increased intestinal problems. Prior to 2006, NE was not considered a major poultry disease [78], but in subsequent years, it has become one of the most significant and costly diseases in poultry production [79]. This has resulted in a substantial increase in the demand for therapeutic antibiotics to treat the disease [80]. NE, caused by the gram-positive anaerobic bacterium *C. perfringens*, leads to high economic costs and significantly compromises animal welfare in poultry [81]. Another major intestinal disease affecting poultry is coccidiosis, which is a parasitic disease caused by *Eimeria* spp. [82]. Both diseases represent a major global challenge in the poultry industry, causing intestinal dysfunction and associated economic losses [61]. Numerous studies have reported the effects of PFAs in controlling enteric infections and coccidiosis, improving animal immunity, enhancing gut function, and preserving intestinal integrity [68, 83, 84]. Additionally, various PFAs are known to have beneficial spasmolytic, laxative, or antifatulent effects on the gastrointestinal system [85].

Ducetelle et al. [86] reported that intestinal health is determined by host factors such as immunity, mucosal barrier, nutrition, microbiota, and environmental factors and that intestinal problems are associated with imbalances in the intestinal microbiome composition (dysbiosis) as well as mucosal barrier leakage and/or inflammation. The intestine provides a natural habitat for a large and dynamic community of microorganisms that not only regulate their own functions but also influence the systemic functions of the host [87, 88]. Moreover, the intestine contains a major barrier that separates the largest interface between the external and internal environments, while allowing the absorption or secretion of molecules [88–90]. The intestine and its complementary

components affect the host's ability to perform normal physiological functions and maintain homeostasis. These underlying components of intestinal health include effective digestion and nutrient absorption, a stable intestinal microbial population, the structure and function of the intestinal barrier, and effective functioning of the immune system. Paiva et al. [91] reported that intestinal morphology, including villus length, crypt depth, and ratio, affects intestinal health and integrity. A short villus height results in poor absorption and digestion, which can be attributed to a smaller absorption area and the presence of less-mature erythrocytes. A greater crypt depth indicates faster cell loss, which may cause epithelial cell loss, inflammation, and abrasion. Increased villus height and reduced crypt depth resulted in a significantly increased villus height/crypt depth ratio, indicating the presence of mature erythrocytes and the effectiveness of nutrient absorption [67, 91]. Indeed, intestinal morphology can be improved through the use of PFAs. On the other hand, poultry lives in environments where they are potentially exposed to pathogenic agents because of the water and feed they consume and their contact with each other and air. Therefore, the likelihood of a chicken being disease-free is suspected. Healthy animals are those that can perform physiological functions and meet performance standards. This indicates that animals can maintain intestinal homeostasis and can withstand environmental stress.

All animals provide habitats for communities of organisms consisting of commensal and symbiotic microorganisms. There are approximately 100 times more bacterial cells in the intestine (about 10^{14}) than the host cells (about 10^{12}) in chickens. Therefore, intestinal microbiomes act as additional complex organs that contain more than a million genes in the host body. For example, the chicken microbiome contains 40–50 times more genes than all chicken genomes [87, 92]. These microorganisms take nutrients from the host body (or the host's diet) and provide essential nutrients to the host. Additionally, they improve the intestinal environment, enhance gut integrity, suppress pathogen competition, regulate host immune system functions, and control physiological inflammation to maintain immune system homeostasis [93]. Studies on gut health and microbiota in the intestine have emphasized the importance of microbiomes in maintaining intestinal health and normal intestinal function. In germ-free animals (e.g., chicks), both the mucosal and systemic immune systems are underdeveloped, and these animals do not produce normal oral tolerance to dietary proteins. However, these deficiencies can be overcome if conventional or defined microbiota are later colonized in these animals. This indicates a clear link between the development of a normally functioning immune system and microbial colonization in the intestine [94]. Additionally, Nurmi and Rantala [95] have emphasized the critical role of normal intestinal microbiota in preventing unwanted microbial colonization by showing that healthy adult chicken gut contents greatly increase resistance to *Salmonella* infection. The host microbiome is easily altered by diet, pathogen infection, and other environmental factors, unlike the genome; however, in later life, the microbiota is resistant to change. Therefore, these factors play critical roles in digestive physiology, animal productivity, and health. In conclusion, to understand intestinal health, it is necessary to elucidate the interactions among these components as well as the scope of the area encompassed by gut health.

1.4 Effects of phytogetic feed additives on antioxidant defense system and oxidative stress

Poultry inherently has constant interactions with the external environment. They encounter numerous biotic (fungi, bacteria, and viruses) and abiotic

(temperature, radiation, and water) stress factors within their environment that can negatively affect their growth and development [96]. In particular, heat-related stress can lead to economic losses in the poultry industry, as it causes insufficient growth performance, immunosuppression, high mortality rates, reduced reproductive performance, and impaired meat quality [97]. Heat stress occurs when the heat generated by an animal exceeds the heat that the animal can dissipate into the environment. This imbalance can be caused by variations or combinations of environmental factors (e.g., sunlight, thermal irradiation, air temperature, humidity, and movement) and animal characteristics (e.g., species, breed, and metabolic rate) [98]. Animals experiencing heat stress tend to reduce feed intake to decrease heat production, which negatively affects growth performance. Therefore, heat stress has long been a major concern among both scientists and poultry farmers, particularly during the spring and summer months in arid and tropical regions where temperature increases. Broiler chickens are chronically exposed to high temperatures, especially during the finishing phase, leading to chronic heat stress [99, 100]. Chronic heat stress can be categorized as periods of high ambient temperature for a certain duration during the day, followed by normal temperatures for the rest of the day over an extended period (days to weeks). Ambient temperatures above the thermoneutral zone of broiler chickens are associated with oxidative stress [101]. Improving the adaptation or resilience of animals to stress primarily depends on understanding the effects of oxidative stress agents.

Oxidative stress is defined as the presence of reactive oxygen species (ROS) that exceeds the antioxidant capacity of animal cells. Many radicals and metabolic substances have been identified as potentially toxic and are referred to as “reactive oxygen/nitrogen/chlorine radicals” [102]. These substances are highly reactive and can alter numerous biologically important cellular molecules such as proteins, lipids, and nucleic acids (DNA and RNA) [103]. These radicals contribute to the development of several metabolic dysfunctions, including “oxidative stress” and “oxidative damage” and damage [102]. Oxidized molecules draw electrons from other molecules, causing a chain reaction that can result in widespread tissue damage. Previous studies have demonstrated a relationship between oxidative stress, intestinal epithelial barrier permeability, and gut integrity [104]. Studies have demonstrated a relationship between oxidative stress and intestinal epithelial barrier permeability and gut integrity [104]. Tight junctions (TJs), the fundamental components of the physical intercellular barrier, exhibit variations in their presence and functionality under different physiological and pathological conditions [105, 106]. Junctional adhesion molecules, claudin (claudin-3) and occludin protein families are structural transmembrane TJ components that have the potential to mediate cell–cell adhesion [106]. Within TJs, claudins are the main determinants of selective pore properties [107], whereas the role of occludin in barrier function is more diverse [108]. In contrast, stress that is more severe or prolonged than acceptable for mitochondrial homeostasis can lead to reduced ATP synthesis, cellular calcium dyshomeostasis, and alterations in mitochondrial permeability, all of which render cells prone to apoptosis or necrosis. Apoptosis is a programmed, gene-regulated event in which cells self-destruct, requiring RNA, protein synthesis, and energy, and is essential for maintaining homeostasis. Cells exposed to oxidative stress receive an apoptotic signal and initiate a series of metabolic and physiological processes for self-destruction. Stimulated cells detach from their environment, have severe connections with neighboring cells, and thus disrupt intestinal integrity [109].

Stress can negatively affect the quantity and quality of broiler chicken meat by causing physiological and biochemical damage. Studies have reported higher levels

of dull, soft, and exudative meat, which results in lower color stability and shorter shelf life [110]. Furthermore, heat stress can cause respiratory alkalosis in poultry by disrupting the acid–base balance and intermittent breathing under stress [111]. This was due to the excessive use of H⁺ ions during overbreathing. As a result of using extra H⁺ ions, the blood pH increases, and poultry can die as they are unable to control their pH. However, stress increases water consumption in animals, leading to a greater than 60% increase in fecal water content and excessive water loss in animals [112]. Stress can also impair the intestinal barrier function [113, 114]. This is due to the redistribution of blood flow to vital organs, such as the brain and heart, which damages sensitive intestinal tissues and villi [115, 116]. This leads to inflammation and increased permeability of the intestinal wall [117]. Dehydration occurs when the body cannot retain the necessary water, resulting in greater water loss through the feces. This causes wet litter problems and performance decline owing to malabsorption. Stress also alters blood metabolite and hormone levels [118]. Endocrinological changes induced by chronic heat stress have been reported to stimulate lipid accumulation in broilers through increased lipogenesis, decreased lipolysis, and increased amino acid catabolism. Heat shock proteins (HSPs), also known as stress proteins, are a group of proteins found in all life forms and all cells but are present at high levels when cells are exposed to high or low temperatures or different stress factors [119]. These proteins are important for both intracellular and extracellular immune functions and play important roles in protein folding and unfolding, association and dissociation, and translocation [120]. The most significant member of these proteins in terms of heat stress is the 70 kDa heat shock protein (HSP70; [121]). These proteins act as molecular chaperones and participate in the folding of proteins into their three-dimensional forms. They are functional in various aspects such as protein folding, cellular regulation, and prevention of the accumulation of inappropriate proteins in the cell under different stress conditions [122]. Heat shock proteins, which have the potential to bind damaged and misfolded polypeptides, prevent their degradation, thereby playing a role in potentially protecting the cell against stress [123].

In the egg-laying poultry industry, as in the broiler chicken sector, researchers are interested in exploring new breeding techniques, improving feed utilization, and enhancing egg quality to achieve forward-looking goals. Enriched cage systems for laying hens have been developed as a result of research efforts aimed at partially addressing the welfare shortcomings of traditional cages and maintaining intensive production [124]. Nevertheless, chickens are still raised in cages. Consequently, laying hens in intensive farming systems must continually cope with stressful factors. Stress can lead to increased ROS levels in chickens, lipid peroxidation, and consequently, a decline in egg quality. This decline in egg quality can cause significant economic losses in the egg-laying poultry and hatching-egg industries. Moreover, stress can result in various types of damage in chickens, such as protein oxidation, nucleic acid damage, enzyme inhibition, apoptosis activation, and cell death. Stress in chickens also has numerous physiological consequences, including increased body temperature, reduced voluntary feed intake, immunosuppression, changes in electrolyte balance and blood pH values, impaired endocrine and reproductive functions, decreased cellular energy availability, changes in the digestibility and metabolism of various nutrients, alterations in the structure and function of the intestinal epithelium, changes in the normal and protective microbiota, and increased circulating cortisol and corticosterone levels. However, detoxification and control of these free radicals are necessary for poultry to continue their lives under these conditions and maintain their ideal yield and performance.

Poultry possess molecular defense mechanisms to reduce or prevent these adverse effects. These response mechanisms can be grouped into three categories: homeostasis of macromolecules and ions, the synthesis of protective molecules, and ROS formation and detoxification. Homeostasis of macromolecules and ions is one of the primary response mechanisms of cells to dehydration. In animals, another response to stress is based on the synthesis of protective molecules, such as low-molecular-weight soluble substances, osmolytes, and heat shock proteins. These molecules act as osmotic regulators and osmoprotectants within the cells. The final molecular response to stress involves the formation and detoxification of ROS, for which both enzymatic and nonenzymatic antioxidants are responsible under stress conditions. The main ROS in cells are singlet oxygen ($^1\text{O}_2$), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\text{OH}^\cdot$), which under normal conditions are constantly balanced within the cell and function as signaling molecules and regulatory compounds [125]. In other words, it expresses the oxidative state, defined as the overall balance of oxidation and reduction reactions within a cell or tissue. The oxidative state involves a balance between free radicals and antioxidants and is crucial for the healthy functioning of cells. The oxidative state can also affect the redox state, a concept representing the balance of oxidation and reduction reactions in cells and the ratio of electron acceptors and donors (reductants and oxidants) within the cell. The redox state is necessary for cellular energy production, signal transmission, and other vital processes. However, under stress conditions, ROS production increases in cells, disrupting oxidative balance. Oxidative stress occurs in animals when antioxidant defense systems fail to neutralize ROS or when free radical production is excessive.

Poultry contains various antioxidants that control and detoxify ROS to sustain their lives and cope with oxidative stress. Antioxidants are substances that can oxidize at low concentrations and reduce or inhibit the oxidation of other substrates (through electron transfer), thus combating oxidation. Nonenzymatic antioxidants include ascorbic acid (AA), tocopherols (vitamin E), carotenoids, glutathione, and phenolic compounds. Enzymatic antioxidants include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT). The cellular localization and roles of enzymatic and nonenzymatic antioxidants differ. Indeed, PFAs are also known as excellent natural antioxidant sources and contribute to meeting the daily antioxidant needs of poultry and protecting against oxidative stress damage [126]. The effects of PFAs on the antioxidant defense system and oxidative stress can be summarized as follows: (1) Antioxidant effect: PFAs possess strong antioxidant properties through the secondary metabolites they contain, neutralize ROS, and reduce cell damage. (2) Effects on enzymatic antioxidant defense systems: PFAs can enhance the activity of antioxidant enzymes, such as SOD, CAT, and GPx, helping combat oxidative stress. (3) Effects on cellular antioxidant defense systems: PFAs can increase intracellular antioxidant capacity, such as glutathione, thereby protecting cell membranes and proteins from oxidative damage. (4) Reduction of inflammation: PFAs can control inflammation by reducing the production of inflammatory mediators and cytokines, thereby contributing to the fight against oxidative stress. (5) Modulation of stress response: PFAs can help reduce the effects of oxidative stress by modulating stress response. They can achieve this by regulating the expression of genes associated with cell and oxidative stress. In conclusion, phytogetic feed additives play a significant role in improving animal health and productivity by supporting the antioxidant defense system and by demonstrating positive effects on oxidative stress. Previous studies investigating the efficacy of PFAs on the immune system, antioxidant defense system, intestinal microbiota, and morphology of poultry are summarized in **Table 3**.

Animals	Treatment groups	Major results	Reference
1-day-old Ross 308 male broilers	Five treatment groups: 2 basal diets (control), or diets with 250, 500, 1000, and 2000 mg/ kg plant extract blend (PEB)	↓ Total bacterial and coliforms count in PEB-supplemented groups, compared to the control, ↑ Lactobacilli count at 500 mg/kg and higher levels of PEB groups, Duodenum, ↑ VH in 500 and 1000 mg/kg supplemented groups, ↓ CD in higher levels of PEB groups, ↑ highest V/C rate at 1000 mg/ kg PEB level, ↑ the highest jejunal and ileal VH and V/C rate in the 500 mg/kg PEB group, compared to the control.	[127]
1-day-old Cobb 500 broilers	Four treatment groups: basal diet (control), basal diets supplemented with 3 g/kg <i>Embllica officinalis</i> (Amla), <i>Cichorium intybus</i> (Chicory), and <i>Allium porrum</i> (Leek) extract	↓ Meat cholesterol, triacylglycerol, and serum total lipids, triacylglycerol, cholesterol, white blood cells, phagocytic index, phagocytic activity, eosinophil, and lymphocytes count in groups supplemented herbal extracts, ↓ MDA, catalase, C-reactive protein (CRP), serum amyloid A (SAA), transferrin (TRF); ↑ glutathione peroxidase (GPx), and superoxide dismutase (SOD) in supplemented groups, ↓ Liver and kidney urea, creatinine, AST, ALT, and lactate dehydrogenase (LDH) by herbal extract groups compared with the control.	[34]
2-days old Japanese quail	Three treatment groups: basal diet (control), basal diet + <i>Lippia gracilis</i> Schauer essential oil (LGSEO, 400 mg/ kg), and basal diet + chemical antimicrobial (500 mg/kg of diet)	→ Intestinal content of <i>Escherichia coli</i> , <i>Salmonella</i> spp., <i>Staphylococcus</i> spp., <i>Lactobacillus</i> spp. by LGSEO-supplemented compared to the control, ↓ The concentration of thiobarbituric acid reactive substances (TBARS) in the liver by LGSEO treatment, → Alanine aminotransferase (ALT), AST, uric acid, and creatinine in the LGSEO group.	[128]
21-weeks- old Roman laying hens	Four treatment groups: Basal diet (control), or diets with encapsulated essential oils and organic acids (EOA) at 150, 300, and 450 mg/kg	At 25 and 30 weeks, → Duodenum, jejunum, ileum VH, CD, and V/C by EOA treatment, whereas ↑ jejunum intestinal wall thickness (WT) in supplemented with 150 mg EOA/kg diet compared to the control, In duodenum, ↑ aminopeptidase activity in the EOA300 group, and maltase activity in the EOA400 group, → sucrase activity in EOA-supplemented groups at 25 weeks, In jejunum, → aminopeptidase and maltase activity by EOA treatment, ↑ Sucrase in the EOA300 group at 25 weeks, At 25 and 30 weeks, → <i>Lactobacillus</i> , <i>Bifidobacterium</i> , <i>E. coli</i> , and <i>Salmonella</i> by EOA in ileum, ↓ ileum and jejunum immunoglobulin A in EOA-supplemented groups at 25 and 30 weeks, respectively, ileum immunoglobulin A in EOA-supplemented with 150 mg/kg compared to the control.	[129]

Animals	Treatment groups	Major results	Reference
60-weeks-old Hy-line Brown laying hens	Four treatment groups: Birds fed a basal diet (control) or basal diets supplemented with oregano EO at 100, 200, and 400 mg/kg (EO100, EO200, and EO400)	↑ the abundances of Burkholderiales, Actinobacteria, Bifidobacteriales, Enterococcaceae and Bacillaceae, ↓ <i>Shigella</i> abundance in the ileum in the EO200 group compared to control. ↑ Chymotrypsin, → α-amylase and lipase activity in dietary oregano EO addition groups, → villus height (VH), crypt depth (CD), villus height to crypt depth ratio (V/C) following oregano EO treatment.	[130]
60 weeks old Qjiling breeder hens	Three treatment groups: control group – basal diet, basal diets supplemented with 30, and 60 mg/kg mulberry-leaf flavonoids(MF)	↑ Superoxide dismutase (T-SOD), and → catalase (CAT) activity, ↓ malondialdehyde (MDA) levels by MF treatment in the shell gland of aged hens.	[131]
7 days old Japanese quails	Five treatment groups: D1, basal diet (BD); D2, BD plus palmarosa oil (PO; 100 µg/kg); D3, BD plus lemon myrtle oil (LMO; 100 µg/kg); D4, BD plus α-Tops (mixture of α-terpineol, cineole and terpinene-4-ol; 100 µg/kg); and D5, BD plus cyclodextrin	→ Microbial profile by EOs treatment groups, ↑ VH, and CD, ↓ villus width in groups supplemented with EOs compared to the control in duodenum, ↑ Jejunum VH and villus width in the D2 group, and ↑ CD in D4 group, ↑ Ileum VH in D4 group, villus width in D5 group, → CD by EOs treatment.	[132]
6-weeks-old Laying Japanese quail	Four treatment groups: basal diet (control), or diets with 3%, 6%, and 9% of pomegranate peel powder	↓ Urea and aspartate aminotransferase (AST), parallel to increase in pomegranate peel powder level, → uric acid creatinine levels in the serum after pomegranate peel powder treatment.	[133]

↑, increase; ↓ decrease, → no significant differences.

Table 3.

Effects of PFAs on immune response, intestinal microbiota and health in poultry.

1.5 Effects of phytogetic feed additives on gene expression and protein production

It is well known that the genetic potential of animals and meeting their nutrient requirements are the two primary factors determining the profitability and sustainability of poultry production. Modern poultry production considers both genetics and feeding management to improve economically important traits, such as growth rate, feed intake, feed utilization, reproduction, meat quality, health, and disease resistance. In previous studies, the majority of animal nutrition research has focused on parameters related to the use of specific nutrients to improve animal growth performance, meat quality, and health or to reduce excess waste [134]. From another perspective, conventional nutrition studies have aimed to address nutrient deficiencies that could jeopardize animal health and production performance, based on collected biochemical and cellular physiological information on the roles of diets in

metabolism [135, 136]. However, with the advent of the postgenomic era, it is necessary to consider advanced animal nutrition research to further increase the efficiency of animal production [137, 138]. Indeed, it has been reported that a nutrient is not only a chemical component required for a specific metabolic function but also plays a role in information or signal transmission within the cell, thereby influencing processes involved in genotype–phenotype continuity [135, 139]. Consequently, nutrients can regulate gene expression through both direct and indirect mechanisms [140, 141]. The animal genome consists of deoxyribonucleic acid (DNA), which encodes the necessary information for all life processes, including growth, development, reproduction, and even cell death [139]. Within the genome, genes are small hereditary DNA segments that are transcribed into ribonucleic acid (RNA) and then translated into proteins. DNA is located in the nucleus of every animal cell and all cells, regardless of their origin, contain the same DNA [142]. However, the genetic code is expressed or readable at different times and amounts for each cell type. Constitutive (housekeeping) genes, which code for proteins that are always necessary for the organism's survival, have relatively constant expression levels under all conditions [143]. Other genes, particularly those related to nutrient storage, processing, and metabolism, can be induced by intracellular and extracellular environmental stimuli (e.g., nutrients) and, therefore, can control their gene expression in some cases [139, 143]. Regulation of gene expression and transcription is a fundamental mechanism by which RNA production is coordinated and controls critical events such as development, homeostasis, and responses to environmental stimuli [144]. Hasan et al. [137] reported that although life fundamentally consists of a series of gene expression processes that are genetically preprogrammed, nutrients could serve as a “driving force” for these processes.

Like other nutrients, phytogetic feed additives (PFAs) can alter gene expression and protein synthesis by upregulating or downregulating transcription processes [145]. **Figure 1** shows an example of the interaction of PFAs with genes and the potential mechanisms of action of this interaction on diet-mediated cell development (modified from [146]). In this context, gene expression is regulated by transcription factors (TFs) [144]. TFs are proteins that bind to a specific DNA sequence in the promoter region of genes, guide the correct binding of RNA polymerase, and either activate (as activators) or suppress (as repressors) transcription [147]. Recent studies have demonstrated that some nutrients can act as TFs or interact with TFs as ligands (A), alter the concentrations of substrates/intermediates in gene regulation or cell signaling through primary/secondary metabolic pathways (B), and regulate gene transcription by directly modifying signal transduction pathways and signaling (C) [146, 148–150]. Moreover, some researchers have reported that nutrients, including vitamin D, vitamin A, hormones, amino acids, specific fatty acids, and PFAs can trigger gene transcription by binding to nuclear receptors [140]. Consequently, changes in gene expression can affect the stability and activity of the proteins responsible for physiological and biochemical functions in the cell. On the other hand, the field of molecular animal nutrition that investigates the effects of dietary components on cellular gene expression, metabolic responses, and, ultimately phenotypic changes in living organisms is called nutrigenomics [151].

Nutrigenomics, or nutritional genomics, investigates how genes and gene products interact with dietary chemicals to alter phenotypes [134]. In nutrigenomics, nutrients are signals that are sent to specific cells within the body. When a cell interprets information from a nutrient, it sends biochemical information from DNA or genes (genomic science) to RNA (transcriptomic science). This information is then translated into specific proteins (proteomic science) and ultimately determines the metabolic pathways

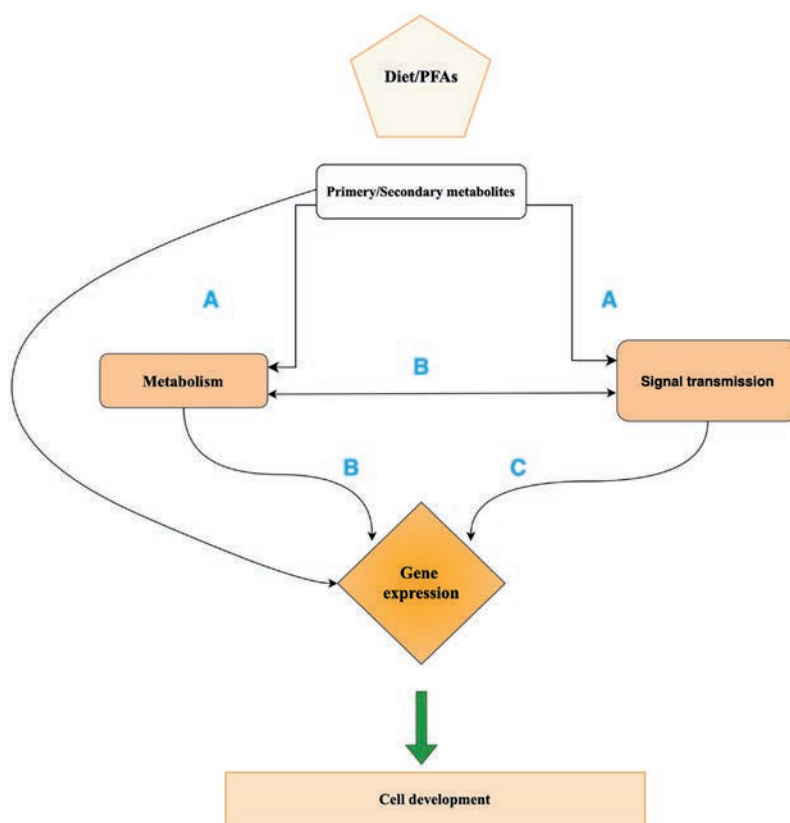


Figure 1.
Cellular gene-diet/PFAs interaction mechanisms.

(metabolomic science) [152]. All biological processes rely on this sequential flow of information, and although they are fundamentally controlled by the genetic structure, external factors such as feed (diet) can influence these pathways [134].

In practice, nutrigenomics can be applied independently or integrated with other “omics” sciences like genomic, epigenomic, transcriptomic, proteomic, and metabolomic through a systems biology approach to analyze cellular and molecular responses to various feed components [137, 151]. Transcriptomics examines all activated RNA transcripts (noncoding RNA, mRNA, rRNA, and tRNA) in a specific cell or tissue at a particular time [153]. Only mRNA carries information from individual genes, serving as a bridge to transmit genotypic messages to a phenotype [137]. The complete set of all transcribed messenger RNA (mRNA) molecules in a cell or tissue is called the transcriptome and represents a snapshot of cellular metabolism [149, 153]. Although there are techniques for measuring the effects of nutrients on individual gene mRNA expression levels, molecular tools such as microarrays (GeneChips) or RNA sequencing (RNA-seq) have been developed to enable the simultaneous examination of thousands of genes. These high-throughput technologies can be used to generate large datasets.

On the other hand, proteomics is a branch of science that investigates the structures, functions, interactions, and modifications of proteins. The entire set of proteins produced by a cell, tissue, or organism at a specific time and under specific conditions is referred to as the proteome. Techniques such as mass spectrometry, two-dimensional gel electrophoresis (2D-PAGE), chromatography, protein microarrays,

Animals	Inclusion rate/PFAs	Major results	Reference
Female Ross 308 broilers	0.2% oregano (<i>Origanum vulgare</i> L.) aqueous extract	A total of 129 genes were differentially expressed in the liver tissue, and there was a major downregulation of genes involved in fatty acid metabolism and insulin signaling pathways in broiler chickens fed the oregano aqueous extract.	[154]
Broilers	Olive mill wastewaters (OMWW)	RNA Sequencing analysis of intestinal epithelial cells revealed that 280 genes were differentially expressed, with the majority of upregulated genes in the OMWW group being involved in the regulation of GO Terms associated with viral genome replication, whereas downregulated genes were mainly involved in cholesterol and lipid metabolism.	[155]
Ross 308 broilers	150 mg/kg capsicum oleoresin (CAP), carvacrol (CAR), cinnamaldehyde (CIN), and their combination (CAP+CAR+CIN)	Compared to the control group, insulin-like growth factor-1 (IGF-1) gene expression in the blood was increased by CAP, CAR, and their combination (18.00, 8.88, and 38.05 fold, respectively).	[28]
Ross 308 broilers	0.5%, 1%, and 1.5% Nettle (<i>Urtica dioica</i>)	Compared to the control group, liver superoxide dismutase 1 (SOD1) gene expression level increased in parallel with the increase in <i>U. dioica</i> level; the highest liver catalase (CAT) gene expression was observed at the 1% level, and glutathione peroxidase 1 (GPX1) gene expression did not change. In addition, the expression of the lung SOD1 and CAT genes also increased.	[156]
Cobb 500 broilers	250 mg/kg grape seed proanthocyanidin (GSPE)	GSPE significantly reduced the serum inflammatory cytokines TNF- α , IFN- γ , IL-1, IL-10, and IL-6 induced by Aflatoxin B1 (AFB1). Similarly, the GSPE + AFB1-treated group showed a significant decrease in the mRNA expression of proinflammatory cytokines (TNF- α , IFN- γ , IL-1, and IL-6) in spleen tissue compared to the AFB1-treated group. In addition, western blotting results showed that GSPE treatment normalized nuclear factor kappa B (p65) phosphorylation and degradation of IB protein induced by AFB1. It was also reported that GSPE enhanced the antioxidant defense system by activating the nuclear factor-erythroid-2-associated factor (Nrf2) signaling pathway. The mRNA and protein expression levels of Nrf2 and down streaming associated genes were upregulated by GSPE addition and downregulated in the AFB1 group.	[157]
Ross 308 broilers	100 or 200 mg/kg free or nanoencapsulated cumin essential oil (EO),	Free EO of cumin (100 mg/kg), free EO of cumin (200 mg/kg), nanoencaps, EO of cumin (100 mg/kg), and nanoencaps, EO of cumin (200 mg/kg) increased the expression level of the jejunum mucin 2 gene (between 1.83 and 1.87).	[158]

Animals	Inclusion rate/PFAs	Major results	Reference
Male Cobb 500 broilers	0.55 g/kg phytogenic feed additives (PFA) such as AV/HGP/16 premix (AVHGP), Superliv concentrate premix (SCP) and bacteriostatic herbal growth promotor (BHGP)	The levels of phosphorylated mechanistic target of rapamycin (mTOR) as well as its levels of mRNA and those of its downstream mediator RPS6B1 were increased in the muscle of the PFA-fed groups. In the liver, phosphorylated levels of acetyl-CoA carboxylase alpha at Ser79, the rate-limiting enzyme in fat synthesis, were induced in the PFA-fed groups. Hepatic expression of hepatic triglyceride lipase (LIPC) and adipose triglyceride lipase (ATGL) was upregulated in the AVHGP-fed group. In the abdominal adipose tissue, the mRNA levels of ATGL and LIPC increased in both the SCP- and BHGP-fed groups. Based on Western blot analysis and gene expression data, they reported that PFA improved feed conversion through modulation of the muscle mTOR pathway and hepatic lipolytic/lipogenic programs, thereby promoting muscle protein synthesis and decreasing hepatic lipogenesis.	[159]
Hy-line Brown laying hens	100, 200, and 400 mg/kg oregano essential oil (EO)	The relative mRNA expression of zonula occludens-1 (ZO-1) in the ileum increased linearly and quadratically with increasing EO supplementation. Furthermore, the relative mRNA expression of interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), and toll-like receptor-4 (TLR-4) decreased in the ileum due to EO supplementation.	[130]
Qjiling breeder hens	30 and 60 mg/kg mulberry-leaf flavonoids (MF)	The protein expression of caspase 3 was downregulated, and Nrf2 was upregulated by dietary MF. Ca transport-related genes (ESR α , ESR β , KCNA1, OPN, CABP-28 K, and CDH6) in the shell gland were upregulated by dietary MF treatment.	[131]
Hy-Line Brown hens	500, 750, 1000, and 1500 mg/kg phytogenic premix (PP, consisting of bioactive substances derived from ginger, lemon balm, oregano, and thyme substances)	In the ovaries in 8th week, the expression of aryl hydrocarbon receptor (AhR) pathway-related genes, including ARNT, aryl hydrocarbon receptor nuclear translocator, CYP1A1, cytochrome P450 1A1; CYP1B1, cytochrome P450 1B1 genes decreased, while GSTA2, glutathione S-transferase alpha 2 gene expression increased in the PP-supplemented groups. In the 12th week, CYP1B1 expression decreased, and GSTA2 expression increased in the PP-supplemented groups. In addition, PP-related cytoprotective potential was demonstrated via beneficial changes seen for the majority of the Nrf2-pathway genes assessed, with the P1000 displaying the most significant differences from the control group.	[160]
Japanese quail	400 mg/kg <i>Lippia gracilis</i> Schauer essential oil (LGSEO)	Small intestinal sodium-glucose cotransporter 1 (SGLT1), glutathione peroxidase 7 (GPX7), and catalase (CAT) gene expression decreased in the LGSEO-supplemented groups compared to that in the control group.	[128]

Animals	Inclusion rate/PFAs	Major results	Reference
Laying Japanese quail	0.1%, 0.2%, and 0.3% <i>M. oleifera</i> seed powder,	In the ovaries, mRNA expression of estrogen receptors (ESR2), follicle-stimulating hormone receptor (FSHR), and steroidogenic acute regulatory protein (STAR) genes increased in the 0.3% <i>M. oleifera</i> group compared to the control group.	[53]

Table 4.
Nutrigenomic studies of PFAs as feed additives in poultry.

and immunoblotting (western blotting) enable the analysis of the quantity, structural changes, protein–protein, protein–DNA, and protein–ligand interactions of a specific protein or thousands of proteins in parallel. Some previous nutrigenomic studies using PFAs as feed additives in poultry diets are shown in **Table 4**. Proteomic studies are crucial for better understanding cell and tissue functions, unraveling disease or performance processes and mechanisms, and identifying new feed additives and biomarkers. The transcriptome and proteome datasets obtained were processed using bioinformatics tools, contributing to the understanding of complex nutrient–gene interaction mechanisms. Consequently, animal nutrition specialists can use these data to create new genotype-specific feeding programs to predict whether animals will respond to phytogetic feed additives or potential side effects and integrate them into diet formulations to increase animal production efficiency in the future.

2. Conclusion

Phytogetic feed additives are products containing natural plant-based components that are added to poultry diets. These additives generally exhibit positive effects on digestion, feed intake, feed utilization, immunity, oxidative status, and stress in poultry. However, the positive effects of phytogetic feed additives can vary depending on their origin, composition, dosage, and incorporation into the feed formulation. Indeed, improper and excessive use of phytogetic feed additives can pose threats to animal health and, consequently, animal production efficiency. Moreover, studies on the effects of phytogetic feed additives on the genome are relatively new. Therefore, selecting and applying the most suitable phytogetic feed additives for poultry should be carefully evaluated, their effects and mechanisms should be thoroughly investigated, and more studies should be conducted to establish specific standards and sustainability.

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Assessment of the Impact of Ungulate Browsing on Tree Regeneration

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Abstract

Ungulates browse on plants and may have an influence on tree regeneration. Browsing percentage (the relative number of browsed terminal shoots) captures little of the effective browsing impact on tree regeneration, such as reduction in stem number or loss of tree species in the future stand. The main objective is to present the most important factors that can influence the impact of browsing and how these factors can be measured objectively. We conducted a literature review of influencing factors, tested these in different areas of the Swiss forest and performed studies to objectively survey them in inventories. Apart from browsing percentage, the following factors are important for estimating the long-term browsing impact: (i) the spatial distribution and density of tree saplings; (ii) the within-tree browsing intensity; (iii) the site-specific height growth of the tree saplings, and thus the time needed to grow out of the reach of browsers and the possible changes in growth rate ranking between the different selected species; (iv) the possible delay in the tree response after browsing; and (v) the tree mortality induced by browsing. The first four of these factors can be assessed easily and should thus be included in future inventories of browsing impact.

Keywords: growth rate, herbivory, tree regeneration, ungulate browsing

1. Introduction

Wild ungulates, such as roe deer (*Capreolus capreolus* L.), red deer (*Cervus elaphus* L.), and chamois (*Rupicapra rupicapra* L.), depend on plants as food. The leaves, shoots, and bark of tree saplings are part of their normal diet [1, 2]. The browsing of terminal shoots (also called leading shoots) can cause a loss of tree height and, depending on the situation, a loss of increment in the following years or even increased mortality [3]. Ungulates thereby selectively browse on a single tree species [4] or even certain individuals [5]. Not all tree species are able to respond quickly and effectively to browsing (see review by [6]). For example, silver fir (*Abies alba*) from high-elevation provenances showed a delayed response to shoot pruning (simulated browsing), whereas Norway spruce (*Picea abies*) did not [7]. Wild ungulates,

therefore, influence the growth of tree saplings differently, depending on the tree species, which can alter their relative abundance and thus their establishment success [8]. Observations, experiments, and model simulations in many temperate and boreal forests have shown that selective browsing by wild ungulates can affect the development of a forest stand and lead to substantial changes in the composition and structure of plant communities [3]; for example, fir may decline [9]. According to the recent enforcement aid on biodiversity in Swiss forests, the degradation of ecosystems as a result of the loss in tree species diversity due to ungulate browsing is now one of the main challenges in mountain forests in Switzerland [10].

In large areas of Europe, the density of wild ungulates has increased drastically in the last decades [11]. With higher ungulate densities, the proportion of tree saplings that are browsed increases [9], as does the amount of damage [12], although not linearly [13]. In Switzerland, the browsing percentages for the most selected tree species (silver fir and oak species) have increased considerably in the recent years [14]. However, the long-term influence of browsing on forest development cannot be derived directly from browsing percentage. Here, we present research results suggesting why this is the case.

In this chapter, we first define browsing percentage and discuss the methods that are frequently used to measure it. We then describe the main factors that determine the influence of browsing on tree regeneration (**Figure 1**). We do this by explaining each factor in turn and suggesting objective measurement possibilities. The factor “tree response after browsing” has no separate section, as it is explained in the sections about within-tree browsing intensity and height loss due to browsing. We conclude our review by suggesting that information on these important factors is necessary to estimate the effects of browsing on tree regeneration and should therefore be considered in future surveys of browsing.

2. Frequency of browsing

The frequency of browsing in an area can be measured objectively and is easily reproducible when quantified as browsing intensity, a specific form of browsing percentage [16]. Browsing intensity is the number of saplings with a browsed terminal shoot in the year preceding the survey (i.e., on the last completed terminal shoot), expressed as a percentage of all observed saplings [16]. Typically, it is assessed for saplings of individual tree species between 10 and 130 cm height since this is the tree size that is browsed most by wild ungulates [17].

Browsing intensity can be determined using different survey methods, mostly with systematically established grids of sample plots. Such surveys are used to determine the presence or absence of browsing on the terminal shoot of tree saplings:

- in fixed circular areas around a sample plot center (e.g., fourth Swiss National Forest Inventory NFI4 [18]),
- in a circular sector defined according to the density of tree saplings (e.g., indicator areas in Switzerland [19] and Austrian wildlife impact monitoring areas [20]), or
- for the k saplings nearest to the plot center, where $k = 1$ (e.g., Swiss NFI3 [21]), or better $k > 1$ (e.g., Bavarian browsing inventory [22] and e.g., [23, 24]; see k -tree sampling [25, 26]).

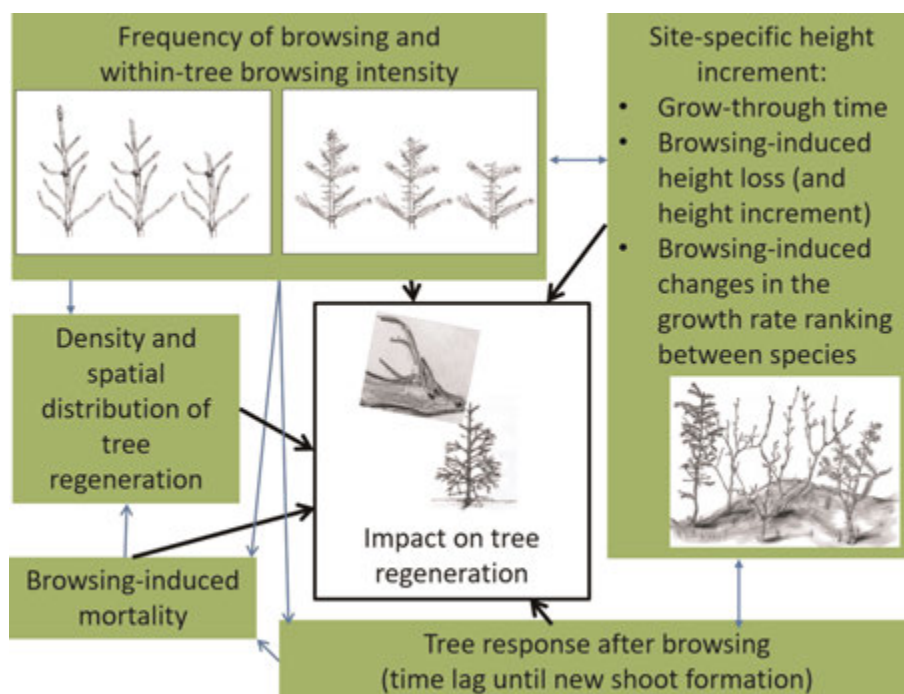


Figure 1.
 The main factors that affect the influence of ungulate browsing on tree regeneration. Adapted from [15].

Statistically, all survey methods are “correct.” Because saplings are irregularly distributed, however, the different survey methods lead to different values for the frequency of browsing. This issue is particularly evident in the case of a clumped sampling distribution [26]. This means that the results of individual survey methods are not directly comparable.

In general, survey methods in which the survey is stopped after a certain number of saplings, regardless of the tree species, or in which only the nearest tree per height class is considered, irrespective of tree species (see also discussion in [27]), are rather inappropriate, as their results depend strongly on the exact survey point, the spatial distribution of the saplings, and the tree species composition. More robust and thus more reproducible results are provided by the complete survey of all saplings per sampling plot, the tree-species-specific termination of the survey after x saplings of a certain species (e.g., 20 per species according to [27]), or the assessment of the k nearest trees to the sample plot center, with k trees defined per species and height class (where $k > 1$).

Since wild ungulates move in space and selectively choose their food, and as favorable areas for tree regeneration are irregularly distributed, the measured frequency of browsing depends on the spatial distribution of the sample plots. The measured values differ depending on whether the plots are sampled in a systematic grid over the entire forest (i.e., in all stages of development and across all forest types) or only in open sites where tree regeneration is more likely [20]. The area (e.g., forest, regeneration area, and problem area) for which a statement on browsing frequency is to be made must therefore be deliberately chosen and communicated accordingly.

3. Within-tree browsing intensity

The wild ungulates in Central Europe are concentrate selectors (roe deer) or mixed browsers (red deer and chamois), and they select not only specific plant species [4, 28], but also certain individual saplings and even plant parts. Particularly with dense tree regeneration, these ungulates select only the (terminal) buds or uppermost parts of the terminal shoot of the most vigorous and dominant saplings [29, 30]. The within-tree browsing intensity (or “browsing strength”) indicates whether only the bud(s), large parts of last year’s terminal shoot, or even older terminal shoots have been browsed away (**Figures 2 and 3, Table 1**). For an individual tree sapling, the within-tree browsing intensity determines:

- the immediate loss in height caused when parts of the terminal shoot are browsed away,
- the subsequent loss of height increment,
- the loss of reserve material necessary for new shoots, and
- the number of meristems remaining on the terminal shoot, that is, the number of regularly formed and dormant buds out of which a new terminal shoot can grow.

Trees whose terminal shoot has only been lightly browsed (end bud bitten off) suffer a smaller loss of height, reserves, and buds than those with heavy browsing. They can also relatively easily grow a new terminal shoot from buds or prematurely sprouted branches (prolepsis) on the remaining part of the terminal shoot [6, 33]. Lateral shoot browsing, on the other hand, does not have a direct impact on tree height or on the number of meristems on the terminal shoot. However, if many lateral shoots are browsed, reserve material in older needles or branches is lost [34], which can reduce the height increase in the following years [35, 36]. If, in an extreme case, all lateral shoots of a tree are browsed, the tree can no longer respond by bending lateral shoots upward, the most common type of response in Norway spruce trees severely browsed at the terminal shoot [7]. For the growth of the individual tree, however, the primary deciding factor is generally whether only the terminal bud or a



Figure 2. Light terminal shoot browsing on silver fir (*Abies alba*; a and b), rowan (*Sorbus aucuparia*; c), and sycamore maple (*Acer pseudoplatanus*; d). only the terminal buds were eaten off, whereas the main part of the last annual height increment—including the lateral buds on the terminal shoot—is still present. These saplings suffer almost no reduction in tree height and can usually form a new terminal shoot without a delay. Photo by A. Kupferschmid.



Figure 3.
Moderate up to heavy terminal shoot browsing on silver fir (*Abies alba*; a and b) and rowan (*Sorbus aucuparia*; c). Almost all of the last year's terminal shoot has been eaten away and with it many meristems. Photo by A. Kupferschmid.

Categories of within-tree browsing intensity	Definition	Example
Not browsed	Terminal shoot not browsed and not damaged	Norway spruce in Figure 5 and current year for silver fir in Figures 5 and 6
Lightly browsed	Terminal shoot lightly browsed, that is, terminal bud eaten off but most of the last height increment, including lateral buds, still present	Figure 2 and previous years for silver fir in Figures 5 and 6
Heavily browsed	Terminal shoot moderately to heavily browsed or even older terminal shoots eaten away	Figures 3 and 7
No shoot	No terminal shoot present because no new terminal shoot formed after browsing in the preceding year or after frost or insect damage in the preceding year	Figure 8
Other damage	Terminal shoot or terminal bud damaged due to reasons other than browsing	Breakage, drought, frost (autumn assessment), insect damage

Table 1.
Categories of within-tree browsing intensity, with short definitions and examples.

long piece of the terminal shoot is browsed [37–39]. The within-tree browsing intensity is therefore a critical variable for assessing the loss of height (increment) and the ability of browsed trees to respond.

In detailed studies on terminal shoot browsing and regrowth in the cantons of St. Gallen and Graubünden in Switzerland, it was found that the terminal shoots of tree saplings were not browsed to the same extent in various forest types [40]. This had an impact on height increment loss in the year of browsing and on the height increments in the years thereafter [40]. The within-tree browsing intensity also affected the relative height increment (see **Box 1**) of the differently browsed tree species (**Figure 4**). For example, in a European beech (*Fagus sylvatica*) forest, lightly browsed

silver fir grew better than Norway spruce, which was never browsed in these forests. Heavily browsed silver fir, on the other hand, fell significantly behind spruce in height growth (**Figure 4**, left). Only in the case of a greatly reduced height increment due to browsing (and/or a considerably delayed browsing response) can browsing be expected to change the growth rate ranking between species and thus have an impact on the entire tree regeneration process in the long term. That is, if lightly browsed silver fir trees grow better than Norway spruce despite browsing, they will not die as an indirect result of browsing. As half of the browsed silver firs in the studied beech forest were only lightly browsed, the number of “substantially” impacted saplings was also halved (**Figure 4**, left). With the classical browsing intensity approach, the impact of browsing on tree regeneration is significantly overestimated in such cases.

By relative height increment, we refer to the length of the terminal shoot formed in the preceding year divided by the tree height. In the case of unbrowsed trees, the terminal shoot length is measured to the base of the terminal bud, while after winter browsing the remaining piece of the browsed shoot is measured.

The ratio of the relative height increments of two tree species can be calculated if an individual of the same height class is present for both species. For example, to calculate the ratio for silver fir and Norway spruce, the saplings closest to the center of the plot are considered. First, the relative height increment is calculated for each of these saplings (considering differences in tree height). Then, for each sample plot and height class, the relative height increment of the more frequently browsed tree species is divided by that of the less frequently browsed species. In the example, the relative height increment of silver fir is divided by the relative height increment of Norway spruce. If this ratio is equal to 1 (gray horizontal line in **Figure 4**), the two tree species have the same relative height increment, that is, they grow equally well in the plots. For values >1 , the silver fir grows better than the Norway spruce in this example, and for values <1 it is the other way round. In the longer term, a ratio clearly >1 or clearly <1 leads to the loss of one of the two tree species. Regarding the influence of browsing, it is useful to know if the ratio falls from >1 or 1 to <1 as a result of browsing, in which case loss of the more frequently browsed tree species is to be expected over the long term. Figures depicting the relative growth rate between species on the y-axis and the within-tree browsing intensity of the selected species on the x-axis (e.g., **Figure 4**) illustrate whether and from which within-tree browsing intensity the browsing has an impact on the selected species and thus on the growth chances of the different tree species.

Box 1.

Relative height increment: definition, calculation, and interpretation.

Compared with beech forests, most of the fir-beech and fir-spruce forests studied by Kupferschmid [40] contained fewer silver fir saplings per sample plot, and their terminal shoots were more heavily browsed. Therefore, most of the browsed silver fir saplings grew significantly less than the unbrowsed ones, though unbrowsed silver fir saplings grew as well as unbrowsed Norway spruce (**Figure 4**, right). This favored spruce over silver fir in terms of height growth [40]. In the longer term, this situation may lead to the dominance of Norway spruce and loss of silver fir. Such a browsing-induced growth difference needs to be assessed with measurements (e.g., comparisons between fenced and unfenced plots, i.e., [41]) to determine if there is indeed a “substantial” effect of browsing on tree regeneration.

When browsing intensity is used as the only measure of the effect of browsing on tree regeneration, such a “substantial” effect is implicitly assumed because only the presence of terminal shoot browsing is considered. While this issue can be studied directly in the forest using “expert assessments,” an objective evaluation based on sample surveys requires information on the within-tree browsing intensity and growth rate ranking of the different tree species. The phenomenon described above (**Figure 4**) did not occur in all of the beech forests or in fir-beech and fir-spruce forests in the study [40], because the within-tree browsing intensity depends on the

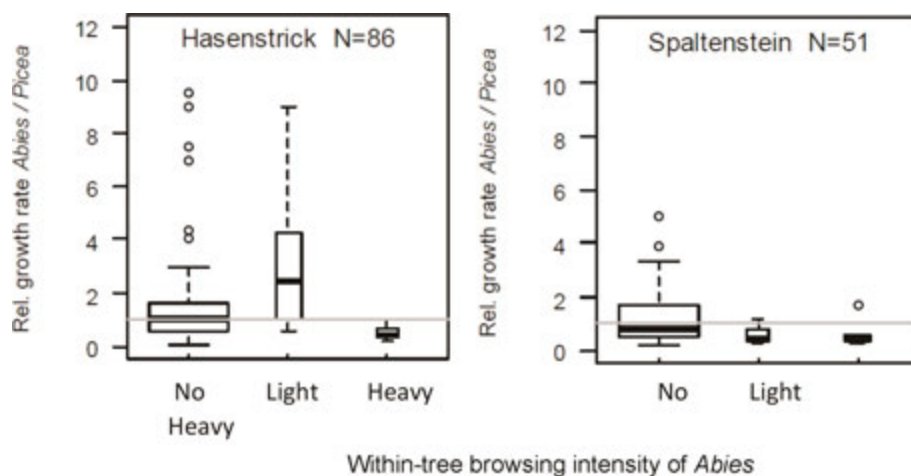


Figure 4.
 Example of results for the ratio of the relative height increment of silver fir (*Abies alba*) to the relative height increment of Norway spruce (*Picea abies*) from two indicator areas (about 20–30 sampling plots) in Switzerland, with 86 (beech forest at Hasenstrick) and 51 (beech-fir forest at Spaltenstein) fir-spruce pairs, ordered by within-tree browsing intensity of silver fir (see box for calculation). Norway spruce were unbrowsed. Box plots feature quartiles (box boundary), median (thick horizontal line), ranging from 10th to 90th percentile (dashed lines) and outliers (dots). The width of the boxes is proportional to the number of measured tree pairs. At the gray line, the relative height increments of silver fir and Norway spruce are equal (ratio of 1). Source: Adapted from supplementary material of Ref. [40].

density of tree seedlings and saplings, tree species, site conditions, stage of development, and likely many other factors [40]. It is therefore important, in our view, to determine the within-tree browsing intensity with surveys.

For both spring and autumn, surveys using four to five categories of within-tree browsing intensity (**Table 1**) have been carried out at various locations and for various stages of development (e.g., [24, 40, 42–44]).

If the number of saplings that are lightly browsed on the terminal shoot and the number of saplings heavily browsed on the terminal shoot are added together and divided by the number of all saplings, the “classical” browsing intensity can be calculated. As described above, however, separation into light and heavy browsing—is helpful for identifying the cases in which the impact of browsing on tree saplings is relevant ecologically or for forestry.

The additional separation of saplings without a terminal shoot (**Figure 8**) provides evidence of the ability of the tree saplings of different tree species to respond after terminal shoot loss. Thus, if there is a time lag until new shoot formation, as often reported for silver fir (e.g., [7, 31, 37]). In addition, the separate category “no shoot” enables a different assignment of these trees to that of the unbrowsed (according to instructions in indicator plots, pers. communication D. Rüegg) or browsed trees (as recommended by Ref. [45]).

The category “other damage” (**Table 1**) gives an indication of further damage (e.g., from frost, insects, rabbits, mice, or lumbering) to trees at a given site and thus represents the relative frequency of browsing versus other damage. For instance, in some places in Switzerland, more frost damage than browsing impact on Norway spruce saplings was found [46].

Suitable methods for assessing within-tree browsing intensity are presented below (see also **Table 2**) and in the next section (**Table 3**). The usual survey of the presence



Figure 5.

Light browsing is not a decisive influencing factor if the lightly browsed species (silver fir in the photo) grows at least as well as or even better than an unbrowsed species (Norway spruce in the photo) occurring at the same site. Photo by A. Kupferschmid.

or absence of a terminal shoot can be refined without much extra effort by assessing the within-tree browsing intensity in categories (**Table 2**).

4. Height (and height increment) loss due to browsing

Site conditions strongly influence the height increment of tree species and are therefore also decisive for the influence of ungulate browsing on tree regeneration [47]. Within-tree browsing intensity may indicate shifts in the growth rate ranking between tree species at a site (**Figure 5**), but only when combined with measurements of the effective annual height increment does it enable estimation of ungulate effects (**Figure 4** and **Box 1**). In survey procedures in which the k nearest saplings to the plot center in each height class are assessed, it is possible to easily integrate measurements of height and height increment (approximated by terminal shoot length; **Table 3** and Swiss NFI4 for an example [18]). As a result of differences in: (i) branching pattern, (ii) visibility of bud scars, and (iii) second and third flushes, the annual height increment may be assessed faster and with less error for certain tree species than for others. In most forests in Switzerland, many of the tree species with height increments



Figure 6.
Silver fir that has been lightly browsed several times on the terminal shoot (within-tree browsing intensity in the last 2 years = “light”). It continues to grow well and thus suffers almost no height loss and no mortality despite repeated browsing. Photo by A. Kupferschmid.



Figure 7.
Ungulate-induced mortality occurs in particular for seedlings that are consumed completely, heavily browsed down to very small residual pieces (image from the experiment by [31]) or are heavily and repeatedly browsed (see films in [32]).



Figure 8. Silver fir (*Abies alba*) without a terminal shoot (“no terminal shoot present” category), presumably as a consequence of earlier heavy terminal-shoot browsing. The trees have grown vertically only through the upward growth of lateral shoots, thus showing a delay of 1 year so far. Photo by A. Kupferschmid.

Tree species	Height class	Within-tree browsing intensity			
		not browsed	lightly browsed	heavily browsed	no terminal shoot present or other damage
<i>Abies alba</i>	1	III		II	
	2		I	II	
	3			III	
	4				
<i>Picea abies</i>	1	III			
	2				I
	3	I			
	4	II			

Table 2. Excerpt from a fictitious survey table for records in circular sample areas, in which within-tree browsing intensity is addressed using four categories.

that are relatively easy to measure are of particular interest for the estimation of browsing-induced species loss, such as *P. abies* (Norway spruce), *A. alba* (silver fir), *Pinus sylvestris* (Scots pine), *Pinus mugo* (mountain pine), *Acer pseudoplatanus* (sycamore maple), *Acer platanoides* (Norway maple), and *Fraxinus excelsior* (European ash). These measurements are more difficult with *Larix decidua* (European larch), *Fagus sylvatica* (European beech), *Quercus petraea* (sessile oak), *Quercus pubescens* (downy oak), *Quercus robur* (common oak), and *Sorbus aucuparia* (rowan). This is particularly true for European beech, due to the frequent formation of second to fifth flushes [48]. In principle, it is acceptable to reduce the height increment

Tree species	Height class	Distance [m]	Tree height [m]	Height increment [m]	Within-tree browsing intensity		Fraying/bark stripping	Insect damage
					Summer	Winter		
<i>Abies alba</i>	1	0.5	12	2.5	0	0	0	0
	1	2.5	10.5	0.5	0	2	0	0
	2	0.8	48	2.0	0	2	0	0
	2	0.9	45	14.0	0	1	0	0
...								
<i>Picea abies</i>	1	1.3	22	0	4	3	0	0
	1	4.5	13.5	3	0	0	0	0
	2							
	2							
...								

Table 3.

Excerpt from a fictitious survey table for recording the nearest two saplings per tree species and height class to the plot center, including the within-tree browsing intensity in five categories (0 = not browsed, 1 = lightly browsed on terminal shoot, 2 = heavily browsed on terminal shoot, 3 = no terminal shoot present, and 4 = other damage on terminal shoot; definitions given in **Table 1**), including a distinction between summer and winter browsing. Here, other characteristics, such as fraying, bark stripping, and insect feeding on each sapling, can be integrated easily. For each individual tree, a single line is filled in.

measurements to one or two preferably browsed main tree species (e.g., silver fir, maple, and ash) and one less frequently browsed species (usually spruce or, depending on the forest type, pine) to estimate the effect of browsing on species-specific height growth (**Figure 4**). For comparisons with beech, measurements in autumn are recommended, as all new flushes have leaves at that point, and thus, the delimitation of years can be seen.

5. Grow-through time: time needed for a tree to grow out of the reach of browsers

If the annual height increment in different tree height classes is known, it is possible to estimate the amount of time during which tree saplings are vulnerable to grazing by wild ungulates. Thus, the approximate grow-through period (“hazard period” according to [17]) can be calculated. If the height increment is small, as in many mountain forests, the trees are browsed for a longer time span than if the height increment is large. This is important for estimating the effect of browsing. If, for example, it takes 10 years for the saplings to grow out of the reach of ungulate browsing and an average of 10% of the saplings are browsed on the terminal shoot per year, a sapling will be browsed, on average, once during this phase (assuming an equal distribution of browsing over time). If the grow-through time lasts 50 years with the same browsing intensity, then a sapling will be browsed five times on average. From this, it is easy to calculate the effect if not 10% but 20% or more of the trees are browsed each year.

The more often a sapling is browsed, the greater the effect tends to be. Consecutive (heavy) terminal-shoot browsing has a greater negative effect than one-time browsing [49–51] because: (i) most of the reserves for the response have already been mobilized in the preceding year and (ii) fewer meristems are available for the response [6]. As explained using the beech-forest example (**Figure 4**, left), the height increment is underestimated at some sites and thus, the grow-through time is Overestimated, if only unbrowsed saplings are considered in the calculation. Ungulates often eat only the buds of the most vigorous, dominant saplings when tree sapling abundance is high. For instance, if red deer or roe deer are offered different trees under experimental conditions, they choose the largest ones and the ones with the most spreading branches [5]. In forests, this choice of the most vigorous plants [52] often results in a situation where the residual shoot pieces of the lightly browsed terminal shoots are longer than the intact terminal shoots of the unbrowsed, less vigorous neighboring saplings [29, 30]. Therefore, the most vigorous saplings grow better than their neighbors despite browsing (**Figure 4**, left, [40]). However, their grow-through time is also prolonged, though not as much as with heavy and frequent terminal shoot browsing [46].

If measurements of height increment are omitted, the grow-through times must be estimated based on separate data in order to estimate the long-term impact of browsing on tree regeneration. This is not easy, as other factors must also be considered, such as light and soil conditions and tree species composition. These influences can be neglected when browsed and unbrowsed trees are measured within the same sample plots. In addition, height increment is also used to estimate the height increment loss due to browsing (see above) and is therefore well suited for estimating the average grow-through time, at least with high sapling densities in all height classes.

6. Density of tree saplings

Not every tree sapling is necessary for sufficient tree regeneration. With 10,000 saplings between 10 and 130 cm in height per hectare of forest, there is a greater chance that some will grow into large trees than with 1000 or only 100 saplings, given equivalent browsing frequency and site conditions (**Figure 9**). With a large number of saplings, there are more individuals in absolute terms that can grow “unhindered” into large trees, due to a variety of factors, such as their particular position (e.g., between tree trunks), their chemical composition, their accessibility, and the fact that they coincidentally have never been browsed, or at least less frequently.

In the study by Ref. [40], within-tree browsing intensity decreased, and thus, the loss of height increment decreased, with increasing density of silver fir saplings, or the density of silver fir saplings was higher at sites with light within-tree browsing intensity. These relationships remain insufficiently explored. However, it seems likely that roe deer, red deer, and chamois are less able to select specific trees when there is little tree regeneration and thus browses more often and more heavily on individual tree saplings (see films in [32]). These repeatedly browsed samplings, therefore, suffer a greater loss of height (and consequently height increment). Conversely, wild ungulates can choose between more trees at higher sapling densities. In addition, when tree saplings are abundant, conditions are often also favorable for other plant species (especially herbaceous plants; [53]), extending further the food choice for ungulates, at least in summer. This in turn reduces the effects of ungulates on tree regeneration.



Figure 9.

If the density of tree saplings is low (as in the foreground of the photo), each sapling is important for the subsequent number of stems in the stand. If, on the other hand, the density is high (as in the gap in the background of the photo), browsing on individual trees is not important, as long as a considerable proportion of the saplings of a target species are not heavily browsed and therefore are not delayed in their height growth compared with unbrowsed tree species. Photo by A. Kupferschmid.

From the point of view of forest services, it is irrelevant how often browsing occurs. Instead, the number of trees that successfully pass the sapling stage is of interest. If, however, we were to record only the trees that passed the sapling stage and pay no attention to browsing, we would not have an “early warning system” [54, 55]. In this case, we would only be able to assess the final outcome and thus would not be able to implement timely measures to protect and promote tree regeneration or to reduce the browsing rate.

Tree density can be reliably determined with surveys involving: (i) counting all saplings in a sample plot of a fixed size (plot-count method), (ii) counting a fixed number of saplings (e.g., 20 or 30 saplings) per tree species, and measuring the “break-off” azimuth of the circle (variable circle sectors), or (iii) measuring the distance to the k nearest saplings per tree species [26]. Which method is chosen is not critical. What is important regarding method (iii) is that the distance to the plot center of at least two saplings per tree species is measured, so that the density of saplings can be determined [25]. The precision (variation) of the results depends mainly on the selected survey radius or maximum search distance [26]. With a 2 m radius, the density is either 0 (if no sapling was found) or ≥ 796 saplings per ha (= 796 if exactly 1 tree was within the 2 m circle), whereas with a 10 m radius 0 or ≥ 32 saplings per ha

would be reported. Therefore, the density estimate depends strongly on the radius or search distance, along with the number of sample plots and the spatial distribution of tree saplings [26].

7. Browsing-induced mortality

Information on browsing-induced mortality is also needed in order to make statements about the effect of browsing on tree regeneration. How many saplings were completely consumed by wild ungulates, and how many were browsed so heavily and/or so frequently that they died? This can be determined by experimentally studying browsing-related mortality. As the basis for their reference value calculations, Eiberle & Nigg [17] assumed, based on fencing experiments, that a silviculturally significant proportion of plants in mountain forests is browsed to the point of mortality when the height increment loss is >25%. However, the browsing-induced height increment loss depends on the site and the within-tree browsing intensity [40]. Therefore, it is also likely that mortality depends on these factors (e.g., **Figure 6**). At the same browsing intensity, browsing-induced mortality is likely to be significantly higher at nutrient-poor sites where tree growth is slow and heavy terminal shoot browsing occurs than at sites with vigorous growth and light within-tree browsing intensity [47]. If seedlings or small saplings are completely consumed (**Figure 7**), they are not considered in the calculation of browsing intensity. Thus, the supposedly moderate browsing intensity at some sites with poor growth may be the result of high browsing-related mortality. This leads to an underestimation of the actual browsing influence when assessments are made on the basis of browsing intensity alone. Data on height increment and within-tree browsing intensity can improve estimates. In order to clearly identify browsing-related mortality, either pair of fenced and unfenced plots should be established or tree seedlings and saplings should be individually marked and repeatedly assessed (see the experiment in Ref. [31]).

8. Conclusion

The long-term effects of browsing by wild ungulates on tree saplings can only be objectively assessed if data is available on (i) the density of tree saplings, (ii) the within-tree browsing intensity, (iii) the height increment of at least the main tree species and thus the grow-through time and the browsing-induced height increment loss, (iv) the proportion of trees with no terminal shoot and thus the time lags after terminal shoot damage, and (v) the browsing-induced mortality (**Figure 1**). At least the first four factors can be easily measured/assessed and should be included in future browsing inventories. The within-tree browsing intensity (i.e., whether only the bud or large parts of the terminal shoot are consumed) can be integrated into any browsing survey with little additional effort. Apart from browsing-induced mortality, all of these factors can be objectively recorded or derived by means of repeated surveys of the k nearest trees to the plot center. The combination of these factors provides meaningful information on how wildlife browsing affects tree regeneration (e.g., [24]).

The aim of the proposals given above is to improve the methods used today to assess the influence of ungulate browsing on tree regeneration. Such an approach would enhance the existing data series on browsing intensity by including additional

measurements or variables that would make it possible to estimate not only the frequency but also the impact of browsing in future surveys.

Large-scale pilot tests of the measures suggested here were carried out in wildlife sectors in the Swiss canton of St. Gallen in spring 2018 [24] and autumn 2022 [43] and in many national parks in Germany in spring 2019/2020 [44]. The authors are willing to accompany further implementations. Of great importance is the continuity of the data series even after the revision of the survey methods, at least for a transitional period, so that comparisons with previous surveys are possible.

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Application of Vermicompost Fertilizer in Aquaculture Nutrition: Review

Sonnia Nzilani Musyoka and Rita Nairuti

Abstract

Semi-intensive aquaculture using ponds is among the most common practices of fish production, whose output depends highly on the ponds' natural primary productivity. With the increased sustainability and health concerns with artificial fish feeds and chemical fertilizers, organic manure has been credited as a cheap, safe and sustainable alternative source of aquaculture nutrition. Apart from supplying nutrients to the phytoplankton, organic manures supply food directly to zooplankton and fish, provide substrate for microbes and improve water and pond sediment quality. Vermicompost fertilizer (excrete of earthworms) has been recognized as a potential pond fertilizer because it has superior nutritional quality (of up to five times), contains microbes, and is in ready-for-uptake form. Besides, the vermicompost contains humic acid, which has antibiotic properties, and promotes fish gut health, stress management, and immune systems. Nonetheless, the application of vermicompost fertilizer in aquaculture is still not a common practice. Therefore, this study reviews the concept of vermiculture *vis-à-vis* pond fertilization and the various utilizations of the vermicompost in fish farming. This is to enable fish farmers to make an informed decision on identifying and selecting proper biofertilizer, which can increase yields and cut costs of production, thus maximizing profits and improving resource utilization.

Keywords: vermiculture, primary production, vermicompost, pond fertilization

1. Introduction

Most of the aquaculture productions in developing countries are practiced in rural areas using semi-intensive fish pond culture systems. This rural aquaculture plays a major role in improving livelihoods, enhancing social equity, advancing gender equality, contributing to global food production (food and nutritional security), and promoting regional economies. Consequently, aquaculture helps curb the high rate of malnutrition occasioned by undernourishment in most rural setups (particularly in Africa) since the superior nutrition in fish improves health by providing food and supporting both mental and physical development and functioning [1]. Besides, unlike some animal protein sources, fish and fish by-products are widely accepted

among various social, cultural, and religious backgrounds [2]. Moreover, in developing countries where people have the highest share of fish protein in their diets, the aquaculture sector plays a vital role in maintaining reasonable livelihoods through food and nutrition provision as well as job creation.

Nevertheless, the significant role played by aquaculture and the increasing demand of aquatic organisms as well as the advancement in science and technology, fish farming in most developing countries is still at infancy stages, being practiced secondary and part-time to agriculture.

Fish feed challenge has been identified as one of the limitations to the commercialization of aquaculture, particularly in Africa. This is because farmers prefer and highly depend on fishmeal as the main protein source due to its superior nutritional properties, palatability, and biological value. Consequently, the majority of the farmers do not achieve their full potential due to the cost, scarcity, and ecological implications associated with obtaining the fishmeal.

The majority of rural fish farming is undertaken in semi-intensive culture systems using earthen (polythene lined or cemented) ponds with limited or no supplementary feeding. Therefore, pond fertilization is taken as a crucial step in pond management to promote phytoplankton production that is the key link to the fish food chain. Studies have considered this old age undertaking as a cost-cutting practice that has the potential of enhancing the fish yields up to 2.8 times [3]. Fertilization is undertaken to improve the growth and reproduction of natural food organisms, which include zooplanktons, phytoplanktons, bacteria, microscopic algae, and insects. These natural organisms in water are eaten directly or indirectly depending on the fish-eating behaviors. For example, the herbivorous fish (such as carps) feed on algae and bacteria biomass, the carnivores (such as catfish) consume zooplanktons and insects, while omnivorous fish (such as tilapia) feed on all.

Therefore, when a pond is fertilized for example using manure, some portion is assimilated or stored by the phytoplanktons, while another share is consumed directly by zooplanktons and fish. Another portion goes to pond bottom where it combines with organic matter to promote the development of the microbial community, which apart from being fed by fish, the bacteria and fungi play a crucial role in ponds of decomposing uneaten fish feeds and toxic wastes [3]. There is also another portion of the manure that is adsorbed by pond sediment whereby it improves bottom soil quality and its water retention properties, and in case of decreased nutrients in the pond, these manures are released slowly back to the water body to promote the plankton development.

The fertilizers used are either organic or inorganic. The large-scale fish ponds are disposed to using inorganic fertilizers thanks to their easy storage and distribution as well as consistent and high nutritional contents. However, their utilization is limited by their scarcity, high cost of obtaining them, and the risks of impairing soil fertility, causing environmental degradation through the pollution to receiving waters, CO₂ generation, and depletion of fossil fuels. Furthermore, chemical fertilizers if not used properly can cause fish toxicity by decreasing dissolved oxygen concentration and increasing total dissolved solutes, alkalinity, conductivity, and free carbon dioxide [4]. Besides, chemical fertilizer application beyond certain levels leads to decreased pond's natural productivity due to self-shielding of sunlight by the upper layer of dense crops of phytoplanktons.

On the other hand, small-scale farmers depend on organic fertilizers from agricultural wastes of crops and livestock animals such as cow dung and chicken droppings. Unlike chemical fertilizers, organic manures are cheap and locally available, contain

organic matter, can be directly consumed by fish, and improve soil structure and water retention. In addition, the straw-like particles in organic manure provide an attachment substrate for microbes to flourish. In Israel, Schroeder [5, 6] demonstrated that organic manure application in fish culture yields three times more than inorganic fertilizer. Nonetheless, organic manure nutritional content is variable and low and recently, they are becoming scarce and relatively expensive to obtain due to competition from crop and fuel production. In addition, cow manure increases biological oxygen demand (BOD) because of the high content of organic matter that consumes a lot of oxygen during their aerobic decomposition often leading to anorexic zones in pond bottoms. Further, livestock manure poses the risks of pathogen transfer to fish.

Therefore, there is a need to adopt economically and ecologically sustainable organic fertilizers such as vermicompost, which has no biosafety concerns. The vermicomposting biotechnology takes advantage of the voracious, polyphagous, fast-growing, and high reproduction nature of earthworms to consume large amounts of organic waste and excrete vermiwastes. The vermicomposting products are earthworm biomass and vermiwastes: vermicompost (solid waste) and vermiliquid (liquid waste). The earthworm biomass is either fed directly to aquatic organisms or used as the protein source in formulating fish diets [7–9]. Nonetheless, the earthworms biomass utilization in fish feed production is limited by processing challenges (handling, harvesting, and gut content evacuation) and the presence of anti-nutritional factors (coelom fluid and chitin), which inhibit uptake, digestion, and assimilation by aquatic organisms [8, 9]. The vermiwastes have also been used in formulating fish feeds; however, their utilization is equally limited by relatively low protein contents and processing impediments [10–12]. In addition, vermicompost utilization is still limited by its efficacy and undeveloped market when compared to compost and chemical fertilizers.

Therefore, this study reviews various applications of the vermiwastes in promoting aquaculture nutrition by improving pond primary production. This is to provide farmers with simple, easily available, and cost-effective fertilizer that can increase fish production and resource utilization without compromising water quality, health standards as well as environmental integrity.

2. Vermitechnology *Vis-à-Vis* aquaculture nutrition

Earthworms are ground-dwelling organisms, which belong to the phylum *Annelida* and class *Oligochaeta*. They are voracious omnivores that feed on dead and decaying organic matter. Consequently, the earthworms just like microorganisms serve the critical biological functioning of nature by ensuring nutrient flow from one system to another, thus reducing environmental degradation. For ages, earthworms have been utilized as agents of vermicomposting biotechnology to sustain soil fertility, reclaim wasteland, and treat organic wastes to a relatively less homogenous and desirable esthetic compound. The vermicomposting biotechnology takes advantage of the voracious, fast-growing, and reproduction nature of earthworms to convert organic waste to highly nutritious compounds [7, 8].

In this biotechnology, the earthworms are made to consume large quantities of organic materials, which undergo a biochemical process in the worm's gut before being deposited as an excreta known as the vermicompost or vermicast or vermiwastes. This complex biological and ecological process of vermicomposting is enhanced by the presence of microorganisms, mucus, and enzymes in the gut of the

earthworms to produce a stable and safe compost that contains and can hold more nutrients for over an extended period of time without affecting the environment. The excreta is rich in humus, which contains micro and macronutrients, antibiotics, vitamins, microbes, growth promoters, and fungal communities [13].

The commonly used omnivorous earthworm species in vermicomposting are *Eisenia fetida*, *Lumbricus rubellus*, and *Eisenia andrei*, which besides being voracious and fast-growing, they are epigeic (surface dwellers), hardy, and easy to culture. The vermicomposting process is undertaken in simple structures using locally available organic wastes. It is desirable that the culture systems have a base (e.g., cemented or layered with polythene paper) that control the earthworms from burrowing, prevent leachates, and simplify harvesting. It is recommended to have the culture systems covered to prevent predators, rainfall, and direct sunlight, while the substrate is regularly sprinkled with water to conserve moisture and regulate the worm's body temperature.

The culture substrates for earthworms comprises a wide range of organic materials that can promote the production of fungi, protozoa, and bacteria that are the highly preferred diets of the worms. Earthworms feed on different food materials, such as livestock manure wood, leave, crop and kitchen wastes, sewage sludge, and agro-industrial waste. The commonly used substrates are livestock manure and kitchen wastes. However, the kitchen wastes of onions and meat should be avoided because they kill fish by reducing pH and suffocation, respectively.

Depending on the culture substrate and worms species used, the vermicompost is usually ready within 2 to 4 months, but the vermiliquid can be harvested on daily basis. There are several techniques of separating the earthworms from the compost but the most convenient one is to, stop sprinkling the substrate with water, introduce light from the top side to prompt the worms to bury downwards, and then collect the upper vermicompost. Otherwise, one can starve the earthworm for few days then introduce a new feed substrate at the top prompting the worms to migrate upward then harvest the vermicompost at the base.

The harvested vermicompost has elevated nutrient levels, which are in ready-to-uptake form by plants (unlike compost that requires curing before use) because they are released relatively easily and faster, thus improving plankton and fish growth performance [14–18]. This is because the earthworms ingest large volumes of nitrogen-laden organic matter, mineralize it in their guts, and excretes it whereby it is stored in the vermicompost in form of nitrate that is more bioavailable to plants when compared to the ammonia form found in conventional compost [19, 20]. This mineralization of nitrates due to vermicomposting and the respiration by earthworms drops the C:N of fresh organic matter to the desired ratio of below 20:1 [12].

Apart from the vermicompost, the vermicomposting process produces vermiliquid, the leachate from the organic waste, and the vermicompost. The vermiliquid (also known as vermiwash or worm tea) just as the vermicompost is an equally nutritious fertilizer because it contains fecal excretion, organic materials, microorganisms, enzymes, earthworms secretions, mucus, and organic acids, and in addition, it contains soluble plant nutrients [21]. Vermiliquid has shown more potential to improve aquaculture nutrition because it further contains cocoons, body parts of worms, small and dead earthworms all that is edible to fish, and some zooplanktons and contributes to more nitrogen to the vermicompost [19, 22]. Besides, the vermiwash is known to contain bacterial biomass, hormones, antibiotics, free amino acids, vitamins (pro-vitamin B and D complex), metabolites suitable for fish growth, feed digestion, disease resistance, and immune boosters [16]. In addition, the vermiliquid

has recommendable proteins contents with the potential of substituting basal ingredients in fish feed formulation [10, 11]. Musyoka et al. [12] demonstrated how a mixture of the three vermicomposting products (i.e. earthworms, vermicompost, and vermiliquid) also known as earthworm bedding has superior nutrition that is capable of economically replacing fishmeal in diets of Nile tilapia (*Oreochromis niloticus*) without compromising growth performance. Both the vermicompost and vermiliquid are highly recommended in organic farming whereby the use of fertilizers, growth regulators, hormones, feed additives, and pesticides are not encouraged.

When compared to the traditional compost and other farmyard manures such as cow dung, horse dung, and poultry droppings, vermicompost has been found to be more nutritious, containing relatively good amounts of carbon, nitrogen, phosphorus, calcium, and C:N ratio as shown in **Table 1**. Nonetheless, the quality of these vermicomposts can be enhanced by using nutritious substrates (preferably a mixture of different substrates) and different species of earthworms. For example Musyoka et al. [12] used mixed substrates of kitchen waste, coffee husks, barley waste, and livestock manure and produced vermicompost with superior nutrition when compared to farmyard manure as shown in **Table 1**. Similarly, when Marsh et al. [24] added shredded cardboard to aquaculture effluent they produced a vermicompost with superior nutrition when compared to Rahman et al. [25] who used cow dung substrate alone as shown in **Table 1**.

Studies have shown vermicompost to contain up to seven times more nutritional and plant growth-promoting value thanks to the faster activation of humus when compared to conventional composting [26, 27]. Besides providing nutrition, the humic acid produced during vermicomposting has the ability to suppress the growth of harmful bacteria and fungi (particularly those responsible for mycotoxins production); thus when consumed by animals, it promotes gut health (for increased nutrient utilization), stress management, and immune systems and controls intestinal diseases [28]. Unlike the compost whereby nutrients such as nitrogen are denatured and microbes die due to high temperatures, vermicompost contains a rich microbial community (that remains unchanged even after drying the vermicompost), which has shown to be direct food to zooplanktons and fish as well as being beneficial in

Nutrients (%)	Vermicompost [23]	Vermicompost [12]	Vermicompost [24]	Vermicompost [25]	Farm yard manure [23]
Substrate used	—	Kitchen waste, coffee husks, barley waste, and livestock manure	Shredded cardboard and aquaculture effluents	Cow dung	
Nitrogen	1.6	1.06	2.7	2.65 ^a	0.5
Phosphorus	0.7	0.35	1.6	2.21 ^a	0.2
Potassium	0.8	0.66	2.7	—	0.5
Calcium	0.5	4.13	8	1.83 ^a	0.9
Carbon	—	12.9	40	8.76	—
C:N ratio	15.5	13.57	15.8	—	3.3

^amg 100 g⁻¹ manure.

Table 1.
 Comparison of the nutritional quality of vermicompost and farmyard manure.

reducing pathogenic bacteria [29]. Kaur and Ansal et al. [30] recommended the development of biotechniques for isolating and harvesting of the beneficial microbial biomass present in vermicompost to be used as biocontrol. In addition, the presence of coelom fluid produced by earthworms (whenever they get agitated) makes vermicompost pathogen-free and goes a long way in protecting fish from diseases [29]. Moreover, the vermicomposting presents stable and mature organic matter rich in humic acids that reduce the harmful effects associated with toxic gases produced by undigested or semi-digested organic manure to fish. In addition, the earthworms reduce heavy metal concentrations on organic matter by baring them in their gut and skin whereby they are slowly broken down into non-toxic forms [31]. Further, the non-thermophilic vermicomposting produces fewer greenhouse gases when compared to traditional composting [32]. Other advantages of vermicomposting over traditional composting are, being a friendly technology that is simple, cheap, relatively faster, can be done indoors using locally available organic materials and using less technical expertise. Correspondingly, studies have reported vermicompost to have great potential of replacing inorganic fertilizer. Sinha et al. [33] reported the vermicompost potential to enhance crop growth by up to 30–40% when compared to chemical fertilizers.

In addition, vermicomposting promotes waste treatment and resource utilization particularly in rural areas whereby cheap underutilized agro-industrial residues are in abundance. Importantly, these residues are not directly consumed by humans (unlike fishmeal), hence cheap and have less competition; however, their utilization in aquaculture nutrition is inhibited by unbalanced nutrition, unpalatability, high fiber, the presence of anti-nutritional factors, and have bio-safety, processing, and ethical issues. Various studies have demonstrated the potential of vermicomposting to vaporize these residues to nutritious earthworm biomass, vermicompost, vermiliquid, and earthworm bedding (mixture of the three) [7, 8, 34]. Besides, vermiremediation has been shown to reduce organic solid wastes by up to 75% faster, BOD and TDSS by over 95% and significantly removing chemicals, heavy metal contaminants, and undesirable gases such as H_2S , NH_3 [35, 36]. With the harvesting and processing challenges of earthworms as well as their bioaccumulation of toxic organic and heavy metals, the utilization of vermicompost in aquaculture has been seen as a suitable alternative [37]. Moreover, the utilization of vermitechnology has also been promoted by the preference to consume organically produced foods, the need to conserve energy, control organic waste pollution, and optimize economical resource utilization strategies.

3. Utilization of vermiwastes in fish ponds

The phytoplanktons, zooplanktons, benthos, microbes, and detritus are natural foods for aquatic organisms. These planktons are the key link to the food chain of aquatic ecosystems as they are the naturally preferred feeds by fish particularly the juveniles. Any artificial food in fish ponds is only required to supplement the deficiency of natural feeds in terms of quantity and quality. Just like most conventional artificial fish feed sources, the planktons have protein contents ranging between 40 and 60%, which is optimal for culturing fish [38]. Therefore, promoting the productions of planktons to adequate amounts can contribute to the overall development of fish in ponds and would mean less or none of the artificial feeds.

With the intensification of aquaculture, the abundance and diversity of planktons are augmented by fertilizer application. Intense fertilization has been shown to yield between 15 and 32 kg/ha/day with no additional supplemental feed in semi-intensive fish farming, translating to 100% replacement of conventional feed [6]. The vermicompost manure has been credited as potential pond fertilizer because it is nutritious enough to supply nutrients to the planktons [17, 30]. In vermicompost manured ponds, Kumar et al. [39] noticed a correlation between improved phytoplankton biomass and zooplankton abundance and diversity, with 68.38, 19.77, and 11.38% occurrence of rotifers, cladocerans, and copepods, respectively. These authors recommended a vermicompost application rate of 5000 kg/ha/year for optimum water qualities. Consequently, the vermicompost has been recommended as suitable manure for nursery pond management to provide zooplanktons that are the preferred feed diets by fingerlings. Habibnia and Bahram [40] observed improved growth and survival of *Rutilus kutum* (*Caspian kutum*) fry as a result of improved plankton abundance after vermicompost fertilization at an application rate of 10,000 kg/ha/year. Similarly, Godara et al. [29] recorded the highest content of phytoplankton and zooplankton at vermicompost fertilization at the same application rate of 10,000 kg/ha/year. Correspondingly, Deolalikar and Mitra [41] recorded increased net productivity of 220.83 C/m/h from 32.03 C/m/h when they fertilized *Labeo rohita* pond with vermicompost still with the application rate of 10,000 kg/ha/year.

On average, the majority of the studies have recommended a vermicompost manure application rate of 10,000 kg/ha/year for optimal water quality parameters, and maximum plankton and fish performance as shown in **Table 2**.

Further, the vermicompost provides food directly to zooplanktons and fish [39]. Though the vermicompost alone might contain low protein value for feeding fish directly, the microbes adhering to organic manures improve their nutrition to contents suitable for the majority of aquatic organisms. Rahman et al. [25] fertilized monosex *Oreochromis niloticus* ponds with vermicompost and observed decreased use of supplementary food (from the traditional feeding rate of 5% body weight to 2%), with increased zooplankton genera and abundance, thus subsequently cutting feeding cost by up to 40%. The authors recorded the highest abundance of planktons and net economic returns of 1889 USD ha/15 weeks in vermicompost-manured ponds (at a fertilization rate of 24,000 kg/ha/year) when compared to control, which had 916 USD ha/15 weeks.

Importantly, vermicompost has been shown to improve aquatic organisms' survival and growth performance without compromising water quality [30, 41, 46]. Kaur and Gupta [45] indicated that vermicompost fertilizer did not alter the physicochemical properties of pond water and improved the growth performance of both planktons and fish. Likewise, Ansal et al. [47] and Kaur and Ansal [30] reported significantly increased dissolved oxygen concentration in fish ponds fertilized with vermicompost when compared to those fertilized with other organic fertilizers.

With the improved plankton biomass, water quality, the vermicompost manure subsequently produces significantly high fish growth performance. After fertilizing ponds with vermicompost, Godara et al. [29] observed significant high growth of catla (*Catla catla*), rohu (*Labeo rohita*), and mrigal (*Cirrhinus mrigala*) at fish ponds that received application rates of 10,000 kg/ha and least performance in those manured with cow dung. In the same way, Kumar and Godara [44] observed two times more growth of Indian major carps in ponds fertilized with vermicompost (at an application rate of 10,000 kg/ha/yr) as compared to the control of pig, poultry,

Vermicomposting substrate used	Parameters tested	Fish tested	Recommended application rate	Author (s)
Cow dung	Water quality, fish growth performance, and plankton biomass	<i>Catla catla</i> , <i>Labeo rohita</i> , and <i>Cirrhinus mrigala</i>	10,000 kg/ha/year	[42]
Cow dung and poultry manure	Water quality, zooplankton production, and growth performance	<i>Cyprinus carpio</i>	15,000 kg/ha/year	[30]
Cow dung	Growth performance	<i>Labeo rohita</i>	10,000 kg/ha/yr	[41]
Water hyacinth (<i>Eichornia crassipes</i>)	Water quality, plankton production, and fish growth performance	<i>Cyprinus carpio</i>	3970.56 kg/ha/90 days	[15]
Water hyacinth (<i>Eichornia crassipes</i>)	Plankton abundance and diversity and fish growth performance	<i>Cyprinus carpio</i>	3970.56 kg/ha/90 day	[16]
Cow dung	Growth performance of fish, water quality, and plankton production	<i>Oreochromis mossambicus</i>	4000.00 kg/ha/90 days	[17]
Solid municipal waste	Water quality, soil retention, and fish growth performance	Cat fish, <i>Clarias batrachus</i>	15,000 kg/ha/year	[43]
Livestock manure	Water quality parameters and plankton biomass	<i>Catla catla</i> , <i>Lo rohita</i> and <i>Cirrhinus mrigala</i>	10,000 kg/ha/year	[29]
Cow dung	Water quality, plankton production, and fish growth performance	<i>Oreochromis niloticus</i>	5550 kg/ha/105 days	[25]
Cow dung, pig manure, and poultry manure	Growth performance of fish	<i>Catla catla</i> , <i>Labeo rohita</i> and <i>Cirrhinus mrigala</i>	10,000 kg/ha/yr	[44]
Cow dung	Growth performance	<i>Cyprinus carpio</i>	10,000 kg/ha/year	[18]
Cow manure	Plankton abundance, growth indices, and survival	<i>Caspian kutum fry</i>	10,000 kg/ha/year	[40]
Cow dung	Fish growth performance and plankton biomass	<i>Catla catla</i>	10,000 kg/ha/yr.	[45]

Table 2.
Utilization of vermicompost in fish ponds.

and cow manure. On the other side, Kaur and Ansal [30] produced the maximum weight gain, yields, and specific growth rate of *C. carpio* after manuring the fish ponds for 120 days with vermicompost at an application rate of 15,000 kg/ha/year and a 2% supplementary diet.

Just like most organic manures, vermicompost is recommended over inorganic fertilizers. Chakrabarty et al. [15] observed significantly high production of plankton diversity and *Cyprinus carpio* fish in vermicompost fertilized ponds when compared to those manured with diammonium phosphate. Also when compared to a mixture of SSP and urea in the ratio of 1:1, vermicompost-manured ponds had superior growth performance of both *Oreochromis niloticus* and plankton biomass [16]. In addition, Ghosh [43] noted that vermicompost-manured ponds had increased water retention capacity than inorganic fertilized ponds in monoculture fish farming. Besides, vermicompost-manured fish ponds have almost double soil moisture retention rate when compared to chemically fertilized culture systems [43]. On the other hand, Chakrabarty et al. [17] found vermicompost-manured ponds to have significantly high phytoplankton production of 3034/L (with increased abundance of *Myxophyceae*, *Chlorophyceae*, *Cyanophyceae*, and *Bacillariophyceae* species) when compared to 2100/L found in chemical fertilizer. The authors noted this increased phytoplankton production translated to the improved growth performance of the test fish, *Oreochromis mossambicus* signifying the natural feeds alone contain the essential nutrients required for fish growth.

On the other hand, the vermiliquid has equally shown the prospects of directly improving plankton productivity, particularly zooplanktons such as *Artemia*, whose mass production is often limited by lack of nutritious feeds, costly and laborious tasks. Vahdat et al. [36] demonstrated the potential of vermiliquid powder to promote the survival, growth, body composition, total carotenoids, and reproduction performance of *Artemia franciscana* in small-scale laboratory cultures.

It is interesting to note that a symbiotic system is developed when vermiculture is integrated with aquaculture; the pond wastes provide feed substrates to earthworms, and in return, the earthworms consume and excrete them as biofertilizers for improving fish pond natural productivity. Studies have recommended vermicomposting biotechnology as a suitable bioremediation technic to treat and vaporize aquaculture wastes to stable and safe organic fertilizers and earthworm biomass, that can be reused for increasing pond primary productions and fish feed production, respectively [24, 48]. Aquaculture waste (principally from intensive recirculating systems) is known to be rich in organic matter due to the elevated nutrients in uneaten feeds and the waste products of the fish. The untreated wastes cause pollution, siltation and its use as fertilizer are problematic because they are susceptible to putrefaction and contains disease-causing agents [48]. Therefore, integrating vermiculture into fish farming is fundamental to not only provide nutrition but also help in the recycling of organic wastes including that of aquaculture itself.

4. Conclusion

Vermicomposting has been recognized as a natural and cheap biotechnique of treating and bio-transforming organic wastes to safe and steady biofertilizers with the potential to promote aquaculture nutrition. This is because vermicompost contains

elevated nutrients, organic matter, microbial biomass, humic acid, and exchangeable cations suitable for supplying nutrients for phytoplanktons, providing food directly to zooplanktons and fish as well as improving immunity, disease resistance, and physiochemical properties of water and pond sediment quality. Besides, the biotechnology is highly considered over the traditional composting because it regulates, improves, enhances, and promotes itself, and it conserves nutrients and microbial communities, involves low or no energy, forms little or no sludge, releases minimal greenhouse gases, and is performed on-site using simple structures requiring minimal expertise. This is particularly significant in developing nations, whereby fish farmers are struggling to break-even (even with many underutilized resources) and have less technical know-how, and their purchasing power of chemical fertilizer and commercial fish feeds is very low. Therefore, integrating vermicomposting into fish-cum agricultural activities can not only improve fish yields but also improve water and resource management, thus promoting sustainable aquaculture, and improving food security and ecological balance.

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Section 3

Animal Reproductive Biology
and Technology

Cryopreservation of Oocyte in Livestock: Principles, Techniques and Updated Outcomes

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Abstract

Many biotechnologies are currently used in livestock breeding with the aim of improving reproductive efficiency and increasing the rate of genetic progress in production animals. The term “cryopreservation” refers to methods that allow biological samples to be frozen and then warmed again without losing their vitality. Cryopreservation is a process that freezes and stores fertilized oocytes for later use, such as gametes, embryos, and primordial germ cells; it is a component of assisted reproductive technology. While some procedures still employ slow-freezing methods, the majority now use vitrification, or extremely rapid freezing, for both oocytes and embryos since it reduces the possibility of harm because there is not as much ice crystal formation as there is with slow-freezing methods. Vitrification has proven to be useful in a variety of applications, including the *in vitro* production (IVP) of embryos in agriculturally significant or endangered animal species, such as pigs, sheep, goats, cattle, etc., after *in vitro* fertilization (IVF) procedures in human embryology clinics.

Keywords: livestock, cryopreservation, oocyte, technology, ARTs

1. Introduction

The genetic variety of many domestic livestock breeds is gradually declining; as a result, it is in the global community's interest to conserve livestock genetics [1]. Ideally, preserving populations as live animals is the preferred method. However, this approach can be costly, and unless the breed serves a productive purpose, its success is unlikely [2]. Hence, *ex situ in vitro* cryopreservation methods have been devised to freeze animal genetic resources in genome/gene banks for the purpose of restoring a specific population later [3]. Substantial advancements have been achieved in the cryopreservation of semen and embryos in various domestic species. However, oocytes exhibit high sensitivity to chilling temperatures, and as of now, a universally accepted protocol has yet to be developed. The establishment of ova banks for the long-term storage of oocytes enables the preservation of female genetic material in an

unfertilized state until a suitable male germplasm is chosen. Additionally, the successful cryopreservation of oocytes not only safeguards genetic material from deceased animals but also facilitates various assisted reproductive technologies [4, 5].

The success of animal oocyte cryopreservation was first reported in the 1980s [6]. Several issues were brought up in the literature regarding the typical development of embryos derived from frozen oocytes. Studies have shown that the development of embryos and fetuses was consistently poor in mice [7–12], rabbits [13], and hamsters [14]. It is evident that cryopreservation procedures unavoidably cause cellular and molecular alterations, leading to a decrease in fertilization rate and embryo development [15]. Previous researchers indicated that two freezing methods, controlled-rate slow cryopreservation, and vitrification, are utilized in the clinical setting for oocyte cryopreservation in animals [16, 17]. The process of slow freezing mainly involves the use of a low concentration of cryoprotective agent (CPA) and slow cooling/freezing rates to control various factors, such as thermal shock, that can lead to cell damage [18]. By slowly reducing the speed of cooling from above zero to below zero, the CPA enables sufficient cellular dehydration, resulting in minimal intracellular ice formation [19].

Both forms of damage can be prevented, or at the very least lessened, by regulating the temperature reduction process and adjusting the cellular environment [20]. To illustrate, the piercing effect of ice crystals can be prevented by rapidly freezing the substance, while the substantial increase in solute concentration within cells due to ice crystal formation can be mitigated by using cryoprotectants [21]. The use of cryoprotectants such as glycerol, ethanediol, dimethyl sulfoxide, ethylene glycol, and propanediol [22]. Although most cells cannot endure the freezing procedure without the aid of a cryoprotectant, it should be emphasized that solely relying on these solutions is inadequate for cell survival post-freezing. The ability of cells to withstand different stresses induced by physical and physiochemical alterations during the process, along with the rates of cooling and warming, as well as the cell type, also plays a crucial role in their survival [23].

Throughout cryopreservation, cells encounter numerous unfamiliar surroundings, including chemical toxicity, osmotic fluctuations, and low temperatures, all of which have the potential to interfere with cell functions and lead to cell death [24]. Several variables, such as factors in species, the age and fertility of the oocyte donor, the stage of oocyte maturation, and the cryopreservation protocols, have been found to impact the efficacy of oocyte cryopreservation [25]. A large variation in oocyte parameters in particular livestock causes difficulties in obtaining a consensus on freezing protocols. Previous researchers reported that species, like pigs, have been suggested to exhibit a reduced ability to freeze oocytes due to elevated lipid levels [26, 27]. The freezing techniques and results in terms of fertilization rate, embryo development, and pregnancy rate after embryo transfer have shown variation among different species and laboratories. Nevertheless, the lack of significant achievements in oocyte cryopreservation hindered the widespread adoption of this method in routine clinical practice for an extended period [28]. During the 2000s, advancements in cryobiology through vitrification techniques paved the way for the successful cryopreservation of functional oocytes, resulting in significant advancements in oocyte cryopreservation programs within clinical settings [29]. A significant randomized trial on oocyte donation demonstrated that the quality of vitrified oocytes was comparable to fresh oocytes in relation to pregnancy outcomes [30]. This review aims to provide an overview of the recent advances in human oocyte and ovarian tissue banking and how these clinical reproductive technologies can be used to support fertility conservation strategies in animals. The focus of this chapter is the foundation of cryopreservation of oocytes and embryos in livestock animals.

2. Methods of cryopreservation

There are two techniques that exist for cryopreserving oocytes and embryos: slow freezing and vitrification. The primary distinctions between these methods lie in the concentration of cryoprotective agent (CPA) employed and the speed at which the sample is cooled during preservation [31]. Nevertheless, a refined and altered iteration of the conventional vitrification process has since been introduced, which employs an exceptionally rapid cooling rate in contrast to the slow freezing or traditional vitrification techniques [32]. This enhanced vitrification technique is referred to as ultrarapid vitrification.

2.1 Slow freezing

Slow freezing was initially developed for the cryopreservation of embryos and oocytes [33]. Slow freezing is the process of gradually chilling a sample over the course of two to four hours, either manually or automatically with the use of a semi-programmable freezer [34]. Researchers reported that the technique was developed during the early 1970s [17, 35] and it has long been regarded as the benchmark for cryopreserving oocytes and embryos in animals [20]. In livestock animals, in this technique, specimens are adjusted to a concentration gradient of CPAs to reduce chemical and osmotic toxicity, and to establish an equilibrium between the factors affecting cell harm [36]. After reaching equilibrium, the specimens are placed into straws and gradually cooled at a rate of 1–2°C per minute until reaching temperatures between –5 and –7°C. Subsequently, they are seeded to start the process of extracellular freezing [37]. Subsequently, the samples are cooled slowly at a rate of 0.3–1°C/min until they reach a temperature ranging from –30 to –70°C [38], and then they are immersed in liquid nitrogen for preservation [39]. After being cooled down to an intense temperature ranging from –5 to –7°C, the cells undergo a gradual decrease in temperature at a rate of approximately 0.3–0.5°C per minute until they reach a temperature within the range of –30 to –65°C [40]. The straws are then placed into liquid nitrogen and left there for the necessary cryopreservation period [41]. This procedure stands out due to its gradual cooling rate, which, when combined with the appropriate concentration of CPAs, guarantees that the formation of ice crystals occurs exclusively outside the cell [42]. This mechanism occurs as a result of the solutes that inhibit the formation of ice crystals in the intracellular medium becoming more concentrated as the temperature decreases and the gradual and regulated loss of water [40]. Excessive solute concentrations that could result in cell death after prolonged exposure to such conditions can be caused by excessively slow cooling, which dehydrates the cell cytoplasm [36]. The optimal cooling procedure, therefore, is a balancing act between preventing the formation of ice crystals and the toxicity of high solute concentrations inside the cell is outlined in **Figure 1**.

2.2 Conventional vitrification

Vitrification serves as a substitute for the traditional slow-freezing technique. The controlled-rate slow-freezing approach of cryopreservation was made easier with the development of vitrification [43]. This technique enables the cell and the extracellular environment to solidify into a glass-like state without the formation of ice crystals [32]. The initial successful vitrification event was documented in 1985 by [44], while [45] showcased ice-free cryopreservation of mouse embryos at –196°C. The protocols

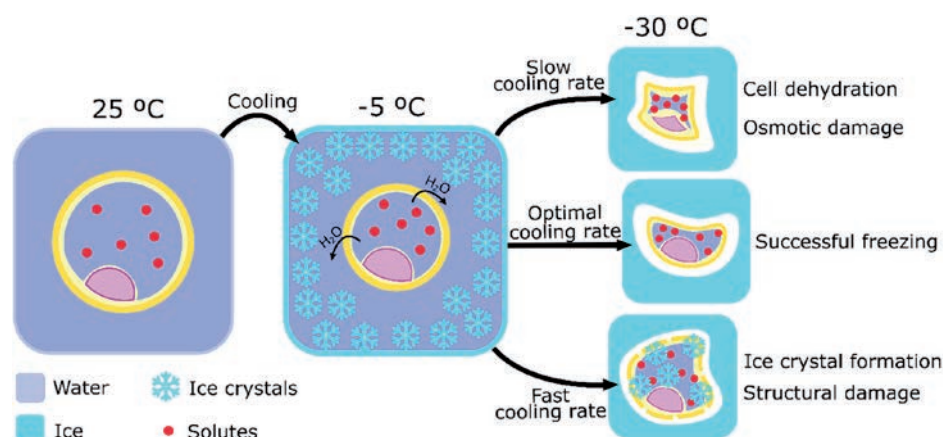


Figure 1.

A diagram illustrating the process of controlled-rate slow freezing and the significance of the cooling rate in cell cryopreservation is presented. The formation of ice crystals commences near the cell as it undergoes a reduction in water volume after initial cooling to approximately -5°C . The relative value of the cooling rate in relation to the optimal rate and the potential harm resulting from the use of inappropriate cooling rates is depicted. A slow cooling rate leads to cell dehydration and osmotic damage due to solute concentration, resulting in chemical harm. Conversely, a rapid cooling rate elevates the likelihood of ice crystal formation within the cell, leading to structural damage [40].

rely on incubating embryos and oocytes in a solution containing various CPAs at elevated concentrations (up to 8 M) [40, 46]. Subsequently, bovine embryos or oocytes are separated into thin straws and exposed to cooling rates of -200°C per minute [47]. Typically, the straws are placed directly into liquid nitrogen at a temperature of -196°C , allowing them to cool quickly and become vitrified [40]. Samples can be preserved in liquid nitrogen for storage [48]. The primary functions of the high concentration of CPAs are to elevate the viscosity of the solution to desiccate the cells and prevent the crystallization of water [49]. This method requires a delicate balance between the harmful effects of CPA and the development of ice crystals within the substance (**Figure 2**). Bailey et al. [50] reported that the reason why this method is commonly used with oocytes and embryos, but not as much with spermatozoa, is due to its previous application. The susceptibility of spermatozoa to the toxicity induced by high concentrations of CPAs is greater than that of embryos and oocytes, primarily due to their heightened sensitivity to osmotic stress [51]. It is possible to reduce the CPA concentration by 50–75% through the use of small volumes (1–1.5 μL) of vitrifying solution [40]. Instant heat release homogeneity is improved after contacting a small amount of sample with liquid nitrogen. Vitrification maintains a liquid molecular structure despite being in a solid state; this prevents ice crystals from forming [52].

The process of vitrification makes it possible to use this technique for livestock animals cryopreserving sperm cells [53]. According to [54], the protocols involve culturing ram spermatozoa in a 0.5 M sucrose solution for a minor shrinkage of the cells, followed by the direct discharge of small droplets of the sperm solution into liquid nitrogen (-196°C). The development of ice crystals, osmotic stress, and toxicity caused by a high concentration of CPAs, this procedure has demonstrated effectiveness in animals' cryopreserving spermatozoa [55]. Despite its promising potential, this technique has not yet been established as a standard protocol for sperm cryopreservation [56].

However, it is unlikely that such a low concentration of CPAs in oocyte and embryo cryopreservation would be effective. This is because the lower surface/volume ratio

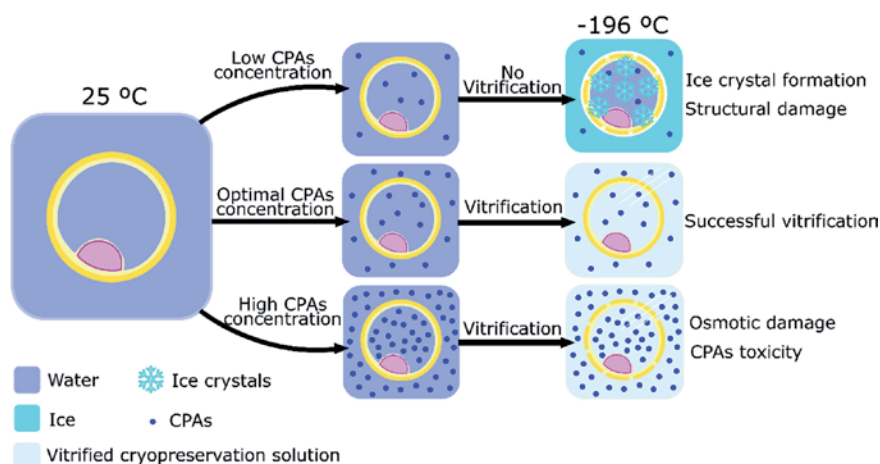


Figure 2.
 A diagram illustrating the vitrification process and the significance of cryoprotectant agents (CPAs) in achieving cell cryopreservation is presented. Varying concentrations of CPAs result in different levels of cell damage post-vitrification. A low concentration of CPAs fails to facilitate vitrification, leading to the formation of ice crystals within the cell and subsequent structural harm. Conversely, a high concentration of CPAs elevates medium toxicity and osmotic damage, causing chemical injury to the cell [40].

of oocytes and embryos compared to sperm cells is associated with a higher likelihood of ice crystal formation [31]. However, for many years, oocytes and embryos have been successfully cryopreserved using vitrification techniques, which involve the use of high concentrations of cryoprotective agents (CPAs). This method has shown a continuous improvement in efficiency over the years [40, 57].

2.3 Ultrarapid vitrification

Ultrarapid vitrification represents an enhanced and updated approach to the conventional vitrification technique as reported by [58]. The previous researcher introduced the concept of ultrafast cooling for cryopreserving oocytes and embryos through their creation of the open-pulled straw [59, 60]. The relationship between the viscosity of the vitrification medium and the cooling rate is inversely proportional [35]. Consequently, a medium with reduced levels of cryoprotectants, with additional additives, can be effectively vitrified at an increased rate of cooling [36]. According to Barbosa et al. [53], achieving vitrification theoretically requires a 1.5 M concentration of any cryoprotectant, if a cooling rate of 15,000°C/min is utilized. The vitrification process enables the use of a low concentration of CPA solution with a very rapid cooling rate, effectively minimizing the toxicity and osmotic stress experienced by the vitrified cells [61]. The ultrarapid cooling rate is accomplished by decreasing the effective volume of the solution to be vitrified [62].

Successful vitrification of mammalian oocytes and embryos using ultrarapid methods is shown in **Table 1**. The findings indicate that ultrarapid vitrification is successful in protecting oocytes and embryos vulnerable to damage from cold temperatures [17]. The open-pulled straw and microdrop methods have also been reported to successfully vitrify bovine oocytes at the metaphase II (MII) stage, leading to the production of live offspring [58]. Booth et al. [67] indicated that full-term young were successfully developed from bovine embryos reconstructed using enucleated oocytes that had been vitrified through the open-pulled straw technique.

Year	Species	Scientific name	Stage	Method of vitrification	References
2000	Cattle	Bovine	metaphase II	Microdrop	[63]
2000	Pig	Porcine	Unhatched blastocyst	Open-pulled straw	[64]
2000	Pig	Porcine	Early blastocyst	Open-pulled straw	[65]
2000	Cattle	Bovine	Morula/blastocyst	Open-pulled straw	[66]
2000	Cattle	Bovine	MII oocyte	Microdrop	[67]
2003	Goat	<i>Capra aegagrus</i>	Cleavage %, blastocyst %, oocytes %.	Cryopreservation	[68]
2010	Goat	<i>Capra aegagrus</i>	Morula and blastocyst stage embryos	Open-pulled straw	[31]
2018	Cattle	Bovine	Blastocyst	Open-pulled straw	[69]
2022	Cattle	Bovine	Metaphase II oocyte	Open-pulled straw	[58]
2022	Buffaloes		Blastocyst	Cryopreservation	[70]
2022	Cattle	Bovine	Blastocysts	Cryopreservation	[69]

Table 1.
Successful cryopreservation of livestock animals' oocytes and embryos by ultrarapid vitrification, resulting in the production of offspring.

Cameron et al. [64, 65] reported that the live offspring have been successfully achieved through vitrification using open-pulled straw in pig embryos, which are highly susceptible to chilling damage.

Oocytes and embryos have been effectively cryopreserved despite being susceptible to factors other than chilling injury. Certain categories of oocytes/embryos, such as pig oocytes, pig embryos prior to the peri-hatching stage, bovine oocytes, and bovine embryos during early cleavage stages, exhibit sensitivity to temperatures below +20°C [71]. Cells that possess a susceptibility to cold temperatures exhibit a dark appearance due to the presence of lipid droplets within their cytoplasm [72]; these lipid droplets are believed to be linked to the occurrence of chilling injury. Therefore, the successful cryopreservation of pig, cattle, and buffalo embryos has been achieved by using embryos at the peri-hatching stage, when there are fewer droplets [70, 73, 74].

3. Principles of oocyte cryopreservation

Cryopreservation oocyte is a valuable technique for preserving germ cells for subsequent uses such as fertilization, as cytoplasts for somatic cells for use in fertilization, as cytoplasts for nuclear transfer from somatic cells to other cells, and for genome banking purposes for valuable animal species. However, Oocytes are highly sensitive to freezing, and the procedure is not as established as in semen or embryos, due to a low permeability to cryoprotectants [75], toxicity, temperature, and time of exposure to the cryoprotectants [76]. While cryopreservation does not affect some processes, it may impair others, like alterations in the oocyte plasma membrane structure and the number of lipids inside the cell in bovines [15] and the cytoskeleton [77]. These processes have been shown to be extremely sensitive to cryoinjury,

which frequently results in cellular disruption and cell death [78]. However, the high concentration of cytotoxic CPAs and/or high cooling rates induce significant cytotoxicity, mechanical, and chemo-osmotic stresses to specimens, causing cellular viability and functional damage, and even cell death [79]. Pared [80] has demonstrated that a number of variables, including the stage of oocyte maturation at freezing, membrane composition, membrane permeability, the kinds of cryoprotectants employed, and the freezing methods, affect the result of oocyte cryopreservation. At prophase I, when the condensed chromatins are shielded by the nuclear membrane, immature oocytes are stopped [81]. Maturation oocytes complete nuclear and cytoplasmic maturation rapidly for fertilization and the ability to support further embryonic development [82].

The cryopreservation of oocytes and embryos involves four main steps, as illustrated in **Figure 3**. In the first step, cells are exposed to a CPA solution to remove water and dehydrate them. By replacing intracellular water with permeable CPA, the freezing point of the cell's content is lowered, reducing the formation of intracellular ice crystals [42]. The second step involves cooling the equilibrated cells to a low temperature and storing them in liquid nitrogen (-196°C). Depending on the cooling method used, this process results in either the formation of small intracellular ice

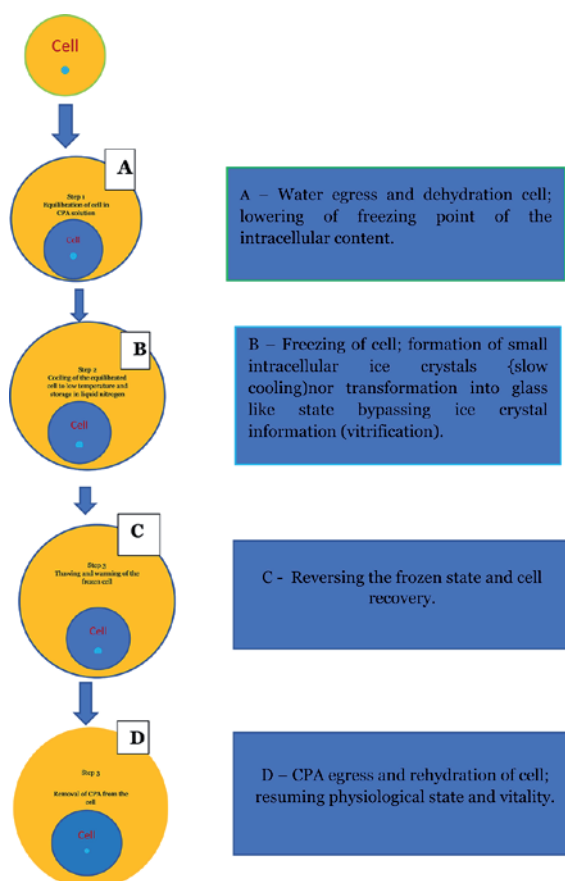


Figure 3.
 Steps involved in cryopreservation of oocytes and embryos [33].

crystals (slow cooling) or the transformation of the intracellular content into a glass-like state without ice crystal formation (vitrification). In the third step, cryopreserved cells are thawed and warmed to reverse their frozen state. After completing Step 4, the cells that have been thawed and warmed are then balanced in the rehydration solution, which facilitates the substitution of intracellular CPA with water molecules. As a result of this crucial step, the preserved cells recover their vitality and resume their regular physiological functions. Steps involved in the cryopreservation of oocytes and embryos are shown in **Figure 3** [33].

The cryopreservation of immature oocytes is superior to that of mature, as they lack a cold-sensitive meiotic spindle [83]. However, the cryopreservation processes itself disrupt the signals responsible for oocyte structure and maturation [84]. Therefore, frozen-thawed oocytes typically have lower rates of maturation and fertilization compared to oocytes that have not been cryopreserved [85]. Problems with oocyte freezing have been attributed to cryoinjury, which can result in issues such as excessive formation of harmful ice inside the cells and chromosome abnormalities [51], disturbance of hyperosmotic stress [86], disruption of actions and microtubules, and zona pellucida hardening [87]. In more recent studies, it has also been suggested that cryopreservation results in alterations in gene and protein expressions in boar [88, 89].

3.1 Cryoprotective agents

Cryoprotective and cytoprotective agents (cytoprotective agents) are fundamental components of the cryopreservation process [24]. The action of CPAs can vary depending on the type of CPA and other factors, including their capability to interact with membrane phospholipids at low temperatures, helping to regulate membrane lipid-phase transition and maintain protein structure and cell integrity [90]. These changes involve alterations in interactions between lipids and lipids, lipids, and proteins, as well as in membrane receptor function, signal transduction, permeability, and transport mechanisms [91]. Therefore, CPAs can be divided into two primary groups: CPAs that can pass through membranes and CPAs that cannot pass through membranes [92]. These substances consist of glycerol, ethylene glycol, propylene glycol (1,2-propanediol), dimethyl sulfoxide, methanol, and butanediol according to [93]. After entering the intracellular fluid, the CPA takes the place of water and disrupts the hydrogen bonding among water molecules, which ultimately decreases the formation of ice inside the cell [94]. Additionally, penetrating CPA also helps increase cellular hydration status during cooling to prevent excessive accumulation of cellular electrolytes [95]. Their relatively small size and amphipathic nature allow them to easily penetrate cell membranes where they can exert their effects. The structure's ability to hydrogen bond with water accounts for much of its protection [22]. The presence of high concentrations of penetrating CPAs in the molar range can induce cellular toxicity or apoptosis by causing osmotic stress or shock [40]. Mammalian cells should be able to tolerate changes in the volume, as they are exposed to being in contact with the hypertonic CPA solution [96]. This occurrence leads to different levels of water activity in various intra and extracellular compartments. If Mammalian cells can exceed the limit of volume expansion at sub-zero temperatures, resulting in cell destruction or apoptosis as a result of Osmotic Stress or Osmotic Shock [97, 98]. It is important to note that certain cell types will need a specific type and the right concentration of CPA to protect cryoinjury during cryopreservation and cooling [99]. Small molecules (such as sugars) arranged into polymers act to increase extracellular osmolality, resulting in cell dehydration, which serves as a stabilizing mechanism; for this reason, nonpenetrating agent

cooling rates are slow [24]. CPAs with high amphiphilic properties and low molecular weight (typically less than 100 Dalton) are frequently organic compounds that can easily penetrate the plasma membrane due to their permeability in animals [40]. Nevertheless, alternative agents can be employed in cryopreservation to protect cells and tissue samples that do not possess cryoprotective properties. These agents function independently of their impact on ice crystal formation and osmotic effects, effectively preventing cellular damage [24]. The common nonpenetrating CPAs are typically less harmful than penetrating cryoprotectants, disaccharides, and polymers, such as ficoll, poly-ethylene glycol (PEG), hydroxyethyl starch (HES), polyvinyl alcohol (PVA), and polyvinyl pyrrolidone (PVP), are examples of such substances [92]. Utilizing a mix of penetrating and nonpenetrating CPAs offers the most effective approach to minimizing cryoinjury in the process of cryopreservation [36]. The regulation of molecular dynamics in cell membranes induced by CPAs is influenced by different mechanisms, which are determined by the specific types of CPAs used [36]. For instance, dimethyl sulfoxide exhibits a higher efficiency in diffusing through phospholipid bilayers when compared to other CPAs [100]. However, at higher concentrations (>7 mol %), DMSO causes the thinning of lipid bilayers, leading to membrane damage and cytotoxicity [40]. Additionally, an alternative suggested mechanism involves colligative properties, specifically the phase diagram alteration in solution, as demonstrated in glycerol [101]. This process could regulate the concentration of salt (NaCl) and modify the formation of crystalline solids that take place during the cryopreservation process [102].

3.2 Cryopreservation technique

The cryopreservation methods for oocytes, sperm, and embryos are typically categorized as controlled-rate slow freezing and vitrification, which is also known as “ice-free” freezing [15]. A programable freezer is necessary for conventional slow freezing to effectively regulate the optimal freezing rate [103]. Throughout the cooling process, the temperature is slowly lowered until it reaches a point below the freezing temperature, resulting in the formation of ice [21]. However, the survival condition of cells is associated with the status of the extracellular solution that results from ice formation [21, 104]. Excessive accumulation of ice inside cells, particularly intracellular ice formation, leads to the disruption of cell structure and function, ultimately leading to apoptosis or cell death [105]. The expansion of ice crystals depends on the rate at which freezing takes place [106]; however, the formation of ice crystals is commonly done to prevent the excessive accumulation of ice during supercooling [107]. By each ice crystal that is formed, the osmolarity of the extracellular solution increases, inducing the outflow of water from the intracellular compartment, due to the increased osmotic pressure [94]. Throughout the slow cooling process, embryonic blastomeres undergo dehydration due to the rising osmotic pressure, which escalates as extracellular ice crystals begin to form following ice seeding [58]. Due to the large size of oocytes and their low membrane permeability to cryoprotectants, most cryopreservation methods necessitate a slow freezing rate to ensure adequate CPA permeability [108]. The permeabilities of oocyte membranes to CPA and cryotolerant substances have been shown to vary between species [109]. Empirical study is often necessary to test freezing protocols before they can be used, despite the availability of theoretical models that can predict the optimal freezing rate. The reason for oocytes susceptibility to low temperatures is due to their sensitivity at different cellular levels, such as the zona pellucida, plasma membrane, meiotic spindles, and cytoskeleton [69]. On the contrary, if the freezing rate is too slow, oocytes will experience significant

dehydration and a combination of relatively low CPA concentrations [16]. Hence, the ideal rate of freezing is a gradual process that strikes a harmonious equilibrium between sufficient cellular dehydration and limited formation of ice within the cells. This method typically necessitates low concentrations of CPA, effectively reducing osmotic shock and CPA toxicity. Vitrification, unlike slow freezing, enables a quick shift from a liquid state to a glassy phase or solidification of water [94]. Vitrification is simplified to develop the process of cryopreservation through the controlled-rate slow freezing method [40]. To obtain this outcome, living cells are first exposed to high concentrations of CPAs (including both permeable and nonpermeable CPAs) before being subjected to deep freezing in liquid nitrogen [18, 110]. Nevertheless, this method results in significant osmotic pressures and chemical harm [111, 112].

3.3 Outcome following oocyte cryopreservation

Oocyte cryopreservation has proven to be a highly effective technique in laboratory animals, particularly in Zebrafish [113] and animals [62]. This success can be attributed to the remarkable tolerance of oocytes to cold stress in bovine [114]. The species has made significant advancements in the technology of producing embryos from frozen oocytes [115]. Cryopreservation of oocytes in Zebrafish and mice is a valuable technique for preserving the genetic material of breeds, including genetically modified animals. Understanding the process of oocyte maturation in Zebrafish and mice is more advanced compared to other animal species. While larger species' oocytes are more susceptible to cold stress than those of Zebrafish and mice, viable offspring have been successfully produced from medium to large domestic animals, such as pigs [116], cows [117], and goat [118], as well as sheep [115].

3.3.1 Pigs

In pigs, successful cryopreservation of porcine gametes and embryos has been very challenging due to their sensitivity to cryoinjuries [119]. The high intracellular lipid content of porcine oocytes and embryos is one of the factors contributing to their low temperature sensitivity in buffalo [120]. Despite the negative impact on embryo development, the elimination of cytoplasmic lipids is harmful [121], improved freezing tolerance and embryo development were observed in porcine oocytes after undergoing partial dilapidation before vitrification [121, 122]. The of survival and embryo development, slow freezing has proven to be ineffective unlike in other species [123]. Due to the high sensitivity of porcine oocytes to cryopreservation, most of the research on porcine oocyte cryopreservation has used vitrification as the method [124, 125]. Vitrification has resulted in the successful birth of the initial piglets [126]. The effectiveness of this method was significantly improved during the latter part of the 2000s [125]. Therefore, experimentation involved testing various modifications to vitrification devices, cryoprotectant types, and procedures to minimize cryoinjuries and cellular alterations in vitrified-warmed oocytes. Significantly, the process of vitrification brings about various changes in porcine oocytes. These changes include disruption of the cytoskeleton [127], chromosomal abnormalities [128], dysfunction of organelles [129–131], oxidative stress [132], disturbance in calcium levels [133], apoptosis [134], and alteration in epigenetic patterns [17]. In recent, the vitrification of porcine oocytes has been linked to changes in transcriptomic and proteomic [135]. Both immature and mature-stage oocytes have been effectively preserved through either conventional slow freezing

or vitrification methods [1, 127]. When immature oocytes are vitrified, there is an improvement in cell cytoskeleton rearrangement and embryo development compared to other methods [100]. Furthermore, research has demonstrated that vitrification can lead to parthenogenic activation at a significant rate of around 50% when oocytes are vitrified at the mature stage [125]. The vitrification process causes a disturbance in cellular functions, particularly in mitochondrial activities, which subsequently elevates the levels of reactive oxygen species. Consequently, this disruption leads to the apoptosis of cells [136]. Hence, the utilization of antioxidants like astaxanthin and caspase inhibitors resulted in a reduction of apoptosis and enhancement in the growth of vitrified warmed in pig oocytes [137, 138].

3.3.2 Bovine

Many reproductive biotechnologies have been utilized for the efficient production of large domestic animals, including pigs and cattle with a high significant economic importance value [58]. Cryopreservation of unfertilized oocytes can be combined with advanced reproductive techniques, along with its potential benefit as a means of preserving female genetic material. Oxidative stress can produce harmful effects on embryonic development, and among these effects, we highlight metabolic alterations such as the depletion of adenosine triphosphate (ATP) levels, changes in ion channels, deleterious effects on protein synthesis, lipid peroxidation, alterations in membrane permeability, and alterations in mitochondrial and ER functions [61, 62]. Therefore, it is important to use cryoprotective additives (CPA), such as dimethyl sulfoxide (DMSO), ethylene glycol (EG), or glycerol alone or in combination, when cryopreserving cells in any methods [58]. Due to both hydrophobic and hydrophilic characteristics, as well as the relatively small molecular weight, these CPAs are permeable to the plasma membrane. The application of vitrification as an alternative to conventional freezing can reduce the equipment required, but the technician-dependent performance of the vitrification process is a limited factor for its widespread use [58]. So far, successful vitrification-producing pregnancy and/or birth of live offspring has been reported with preimplantation embryos from various mammalian species including humans [63]. The method was found uniquely efficient for cryopreservation of pre-compaction stage *in vitro*-produced embryos, which so far are regarded as extremely sensitive to cryoinjuries [64]. Using identical parameters, embryos from the late stages of preimplantation development were also vitrified with high survival rates [65]. The higher lipid content in the *in vitro*-produced embryos has been related to its low cryotolerance [66]. However, the use of delipidant agents during *in vitro* culture does not necessarily result in increased cryotolerance [67]. In addition to the stress of *in vitro* production, it is relevant to mention that the cumulus-oocyte complexes (COC) have their own history when they arrive in the laboratory, which influences subsequent *in vitro* development [26]. Heat stress, for example, is a very common type of stress that affects oocyte and embryo development [70]. However, little is known about how lipid metabolism influences the embryonic development of oocytes under heat stress. However, it may be a good example to elucidate the importance of lipid reserves in oocytes and embryos under stress.

3.3.3 Goats

Oocyte maturation, parthenogenesis, cloning, and the creation of transgenic animals are among the biotechnological processes that demand a substantial quantity of

oocytes [69]. Compared to the freezing of sperm or embryos, the cryopreservation of goat oocytes has proven to be much more challenging [68]. According to [35] oocytes suffer damage at various levels, impacting the cytoskeleton, meiotic spindle, zona pellucida, plasma membrane, and other structures, making them large and vulnerable cells for cryopreservation. Cooling and freezing matured or ovulated oocytes can cause spindle disorganization, loss or clumping of microtubules, chromosome scattering, increased polyploidy during fertilization, and decreased fertility [71]. The level of reactive oxygen species (ROS) during *in vitro* maturation significantly compromises the ability of oocytes to develop up to the blastocyst stage, as is well known [72] vitrification raises the ROS levels in oocytes of other species. Due to ROS playing a crucial role in the apoptotic process, there has been a close correlation between the increased rate of apoptosis and the increased ROS level of vitrified oocytes [73]. Oocytes in prepubertal females are less able to reduce reactive oxygen species (ROS), which damages the microfilament network and inhibits the development of blastocysts [74]. For this reason, the viability of the vitrification process depends critically on the oocyte's quality and nuclear stage [68]. Kharche et al. [69] investigated the impact of propanediol-based GV-oocyte vitrification in goats, demonstrating a noteworthy decline in the rates of vitrification group zygote pronuclear formation and maturation following IVF. After parthenogenic activation, [75] assessed the impact of vitrifying GV and MII goat oocytes, but they discovered no variations in the two nuclear stage-oocyte groups' blastocyst production. Nevertheless, cryotop [76] and open straw [77] were superior tools for vitrifying GV-oocytes in goats.

3.3.4 Sheep

Cryopreservation of sheep oocytes is significant for preserving the genetic potential of female sheep. The procedure involves freezing eggs, and this maintains their viability and fertility. It is crucial for the long-term preservation of sheep's genetic resources. This method advances sheep embryo biotechnology supporting ewe genetic resources [111]. There are two techniques for oocyte cryopreservation which are vitrification and slow cooling. Both have been tested for cryopreservation of sheep oocytes. The slow-cooling method is more susceptible to cold shock than the vitrification method and as such, cold shock must be thoroughly looked out for in slow cooling. During vitrification, oocytes pass through the dangerous temperature zone at extremely high speed [111]. The status of cryopreservation of sheep oocytes is limited due to the lower cryotolerance of sheep embryos compared to other species like cattle. Despite efforts to improve the process, actual results are not as promising as expected and it is mainly due to the cellular and biochemical changes that occur during freezing and heating. Ultrastructural evaluation of vitrified ovine embryos shows significant damage. Fresh embryos exhibit fewer microvilli and less extensive intercellular junctions and there are also more lipid droplets and cell debris [111, 139]. These challenges call for new approaches to achieving cryopreservation goals, with new tools being developed for vitrification and different ultrafast vitrification protocols. These result in higher efficiency and better tolerance of the samples to temperature during the cryopreservation process. Further, methods like manipulation of lipid content in embryos as a potential way to enhance cryopreservation effects are indeed under consideration [139, 140]. The latest technological advancements in sheep oocyte cryopreservation involve the use of alternative methods such as vitrification and ultrarapid vitrification protocols, which provide a more efficient and temperature-permissive cryopreservation process. Additionally, strategies like manipulating the

lipid content of the embryo are being investigated as a promising approach to improve cryopreservation efficiency [141]. Dilapidation i.e., studies aimed at understanding the correlation between oocyte lipid content and the success rate of vitrified-warmed ovine oocytes have suggested that lowering oocyte lipid content may be a feasible strategy for enhancing the survival of cryopreserved sheep oocytes. Originally, the concept of this strategy lies in the fact that ovine oocytes contain a high level of lipids, and these lipids contribute to low cryotolerance and poor cryosurvival rates. A couple of studies by [111, 142] support it to some extent. Thus, while dilapidation may be helpful to enhance the survival rate of cryopreserved ovine oocytes the outcomes are not significantly different because intracellular lipid content is only one factor among many variables that can lead to reduced tolerance to cryopreservation [111, 142]. Cryopreservation of sheep oocytes is a valuable tool for preserving the genetic potential of female sheep and advancing sheep embryonic biotechnology. While there are challenges associated with sheep oocyte cryopreservation, researchers are actively exploring alternative methods and strategies to improve the efficiency and effectiveness of the process. Ongoing research in this field is expected to further enhance our ability to preserve and utilize sheep genetic resources.

4. Prospects

Progress in oocyte cryopreservation has advanced steadily over the years, yielding positive results. However, enhancing outcomes for cryopreserved oocytes through the study of cryobiology remains a complex task. However, the efficacy of oocyte cryopreservation remains significantly lower in comparison to embryos at a later developmental stage, despite utilizing ultrarapid vitrification, which is currently regarded as the most effective technique available. Therefore, the primary obstacle in this domain is to establish uniform procedures for efficient cryopreservation of oocytes and embryos at their early stages. The aim of achieving success in such protocols theoretically should be like that of their non-cryopreserved equivalent. It is clear from the existing knowledge that the capacity of oocytes and embryos to endure the cryopreservation process differs among various species. Developing a universal standardized protocol for all species seems unattainable. Therefore, it is imperative to prioritize the advancement of species-specific optimized procedures for the cryopreservation of oocytes and embryos. Additionally, it will be captivating to witness forthcoming endeavors in the creation of automated devices for the vitrification of oocytes and embryos. The worldwide field of livestock can be revolutionized by the implementation of an efficient and automated ultrarapid vitrification system. In the future, the focus of cryopreservation will be on developing protocols that preserve the structural and functional integrities of oocytes and embryos after freeze-thawing. These protocols should be reproducible across laboratories worldwide. Achieving these targets will lead to the development of standardized and optimized methods for routine oocyte and embryo cryopreservation in livestock.

5. Conclusions

In conclusion, cryopreservation is the process of preserving living cells at an extremely low temperature, typically in liquid nitrogen. It is essential for maintaining and conserving genetically superior germplasm by preserving oocytes and embryos.

The cryopreservation procedure involves three main stages: equilibration of cells with a concentrated cryoprotective agent solution, cooling and storing cells at ultra-low temperatures, and recovering frozen cells after thawing and warming. Cryoprotective substances are crucial for ensuring the viability of cryopreserved cells during processing and storage at very low temperatures. There are two primary techniques for preserving oocytes and embryos: slow freezing and vitrification. Slow freezing involves gradually cooling samples to form ice crystals inside and outside cells, while vitrification rapidly cools samples to create a glass-like state without ice crystal formation. Ultrarapid vitrification is an advanced technique with rapid cooling and warming rates, considered superior to slow freezing and conventional vitrification. Cryopreservation often results in cryoinjuries such as chilling injury, ice crystal formation, fracture damage, osmotic stress, and the development of multiple asters. The extent of cryoinjuries depends on various factors and significantly impacts the viability of cryopreserved cells. The cryopreservation process has adverse effects that affect the survival of frozen cells after thawing, especially when preserving oocytes.

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Conflict of interest

The authors declare no conflict of interest.

Abbreviation

ARTs	Assisted Reproductive Technologies
CPAC	Cryoprotective Agent
COC	Cumulus–oocyte Complexes
DMSO	Dimethyl Sulfoxide
EG	Ethylene Glycol
HES	Hydroxyethyl Starch
IVF	<i>In vitro</i> fertilization
PEG	Poly-ethylene Glycol
PVA	Polyvinyl Alcohol
PVP	Polyvinyl Pyrrolidone
ROS	Reactive Oxygen Species
NaCl	Sodium chloride

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This volume integrates recent scientific advances across veterinary medicine and science. It starts with updates on increased knowledge of bed bugs and chelonians, which is continuously evolving and driven by technological advancements and improved understanding of anatomy, ecology, immunology, and physiology. The book continues with an examination of current developments in animal nutrition, including the application of phytogenic feed additives in poultry, the ecological implications of ungulate browsing on forest regeneration, and the increasing use of nutrient-rich vermicompost in aquaculture. In addition, it highlights progress in reproductive biology and biotechnology, focusing on methods that enhance genetic gain, preserve germplasm, and address infertility to improve reproductive efficiency. Collectively, the chapters offer a comprehensive and contemporary perspective on evolving research that is shaping the future of veterinary science.

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