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PHYSIOLOGY AND REPRODUCTION

Ionophore coccidiostats – disposition kinetics in laying hens and residues transfer to eggs

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ABSTRACT Poultry production is linked with the use of veterinary medicinal products to manage diseases. Ionophore coccidiostats have been permitted for use as feed additives within the European Union (EU) for the prevention of coccidiosis in various species of poultry with except of laying hens. The presence of chemical residues in eggs is a matter of major concern for consumers' health. Despite such prohibition of use in laying hens, they were identified as the most common non-target poultry species being frequently exposed to these class of coccidiostats. Many factors can influence the presence of residues in eggs. Carryover of these class of coccidiostat feed additives in the feed of laying hens has been

identified as the main reason of their occurrence in commercial poultry eggs. The physicochemical properties of individual compounds, the physiology of the laying hen, and the biology of egg formation are believed to govern the residue transfer rate and its distribution between the egg white and yolk compartments. This paper reviews the causes of occurrence of residues of ionophore coccidiostats in eggs within the EU with special emphasis on their disposition kinetics in laying hens, and residue transfer into eggs. Additional effort was made to highlight future modeling perspectives on the potential application of pharmacokinetic modeling in predicting drug residue transfer and its concentration in eggs.

Key words: coccidiostats, food safety, pharmacokinetic modeling, laying hen, drug residue

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INTRODUCTION

Over the past 2 decades, the global poultry industry has seen tremendous growth in production in an attempt to sustain food security and strengthen the global agro-economy (Blake et al., 2020). Coccidiosis is a parasitic disease caused by protozoan parasites of the genus *Eimeria* which may colonize various segments of the intestinal tract of poultry (Chapman, 2017). These parasites impair nutrient absorption and reduce feed conversion efficiency and weight gain or even cause death, as a result of injury to the digestive system (Chapman, 2017; Blake et al., 2020). Because of the severe economic losses associated with coccidiosis in poultry production, disease control and prevention typically include anticoccidial drugs and/or vaccines against coccidia to achieve

sustainable productivity (Chapman, 2017; Kadykalo et al., 2018; Blake et al., 2020).

Anticoccidial drugs are chemical substances that are specifically designed to prevent and combat coccidiosis (Clarke et al., 2014). They are classified into natural substances called ionophores (lasalocid, maduramicin, monensin, salinomycin, semduramicin, and narasin) which are metabolic by-products of *Streptomyces* spp. and *Actinomadura* spp. (Anadón and Martínez-Larrañaga, 2014; Clarke et al., 2014), and synthetic anticoccidial compounds (diclazuril, decoquinate, halofuginone, nicarbazine, and robenidine) that are produced by chemical reactions (Clarke et al., 2014). Ionophore coccidiostats are the most widely used anticoccidial feed additives in poultry production (Kadykalo et al., 2018; Noack et al., 2019). In contrast to the synthetic coccidiostats, ionophore agents have demonstrated a low risk of resistance development. They typically demonstrate some therapeutic efficacy against bacteria that cause necrotic enteritis, and allow for the stimulation of natural immunity by not completely clearing the parasites from the host (Clarke et al., 2014; Chapman, 2017).

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Ionophore coccidiostats have been approved within the European Union (EU) Regulation (Commission Regulation (EC) No 1831/2003) for use as feed additives for the prevention of coccidiosis in target species of poultry (broilers, turkeys, pullets, and minor avian species) at a defined dosage with a maximum content of the active ingredient in feed at a level of 75 to 125 mg/kg (lasalocid), 60 to 125 mg/kg (monensin), 20 to 70 mg/kg (salinomycin), 60 to 70 mg/kg (narasin), 5 mg/kg (maduramicin), and 20 to 25 mg/kg (semduramicin) (Rokka et al., 2013). They are not allowed to be fed to laying hens due to risks of exposing consumers to violative residues in eggs (Goetting et al., 2011; Beyene, 2016). Most laying hens are vaccinated against coccidiosis (Goetting et al., 2011; Anadón and Martínez-Larrañaga, 2014; Chapman, 2017) and only very few veterinary medicinal products have been approved for use in laying hens (Goetting et al., 2011). Nevertheless, laying hens may become exposed to coccidiostats from the environment, through water and feed, or by illicit use of these drugs in the treatment of coccidiosis, and their residues can be transferred into eggs (Van Der Fels-Klerx et al., 2017). Furthermore, classifying ionophore coccidiostats as feed additives to be used without veterinary prescription has caused great challenges in regulating their marketing and monitoring of their residues in edible poultry products (Piątkowska et al., 2012).

The persistent occurrences of chemical residues in food of animal origin, including poultry meat and eggs is a matter of major concern for consumers' health (Mund et al., 2017; Roila et al., 2019; Sobral et al., 2020). Consumption of eggs contaminated with chemical residues can lead to side effects such as allergic reactions, drug-drug interactions, and disruption in gut normal flora (Beyene, 2016; Mund et al., 2017). Therefore, the concentration of residues of coccidiostats in eggs must be as low as possible to a level that is deemed to be safe for the consumers. The safety of the consumers is usually ensured through the establishment of maximum residue limit (MRL) for a particular drug in each edible poultry product including meat and eggs (Piątkowska et al., 2012; Dorne et al., 2013). The capacity of an animal to produce residues in eggs after drug exposure depends largely upon some factors like pharmacokinetic characteristics, physicochemical properties, and the physiology of egg production (Donoghue et al., 1997; Donoghue, 2005; Beyene, 2016). For this reason, pharmacokinetic and metabolism studies are being carried out in these non-target species to predict the drug uptake, residue distribution, and depletion profile of each coccidiostat drug in tissues and eggs after exposure (Donoghue, 2005; Beyene, 2016). Despite the restriction on the use of ionophore coccidiostats in the production management of laying hens, it was observed that they are the non-target species of poultry being most frequently exposed to such feed additives (Kennedy et al., 1998; Olejnik et al., 2011; Chapman, 2017). Moreover, non-compliant egg samples containing violative residues of ionophore coccidiostats are still being reported by

regulatory authorities (EFSA, 2021; Roila et al., 2021). The number of egg samples that must be collected for the monitoring of ionophore coccidiostats in commercial poultry eggs must be at least equal to one sample per 1,000 tonne of annual egg production, with a minimum of 200 samples required to be collected each year, depending on the volume of egg production in each EU member state. Based on the yearly results of the reports on the monitoring of veterinary medicinal products residues and other substances in live animals and animal products carried out by the European Food Safety Authority (EFSA) (EFSA, 2012, EFSA, 2013, EFSA, 2014, EFSA, 2015, EFSA, 2016, EFSA, 2017, EFSA, 2018, EFSA, 2019, EFSA, 2020, EFSA, 2021), it was observed that ionophore coccidiostats were the most frequent contaminants of commercial poultry eggs with a higher frequency of occurrence as compared to the synthetic coccidiostats (Figure 1).

The relatively recent episode of massive eggs contamination with lasalocid in Poland has increased concern about the presence of violative residues of ionophore coccidiostats, (Poultry World, 2018). The event was reported to have occurred when laying hens were accidentally fed with a feed intended for fattening broiler chickens. As a consequence, more than 4,000 tonnes of eggs were recalled from the market (Poultry World, 2018). This work aims at reviewing literature concerning residues of ionophore coccidiostats in eggs within the EU with a particular emphasis on the sources of egg contamination, their disposition kinetics, and residue transfer into eggs. Since novel strategies for predicting and quantifying residues in edible tissues of animal origin are constantly being developed, this work also attempts to highlight future modeling perspectives on the potential application of pharmacokinetic modeling in predicting drug residue transfer and its concentrations in eggs.

IONOPHORE COCCIDIOSTATS: CHEMICAL PROPERTIES AND MECHANISM OF ACTION

Ionophore coccidiostats are natural substances produced as metabolic byproducts of several strains of *Streptomyces* and *Actinomadura* species of bacteria (Anadón and Martínez-Larrañaga, 2014; Clarke et al., 2014). Chemically, they are organic acids with very complicated structures made up of several tetrahydrofuran rings linked by spiroketal moieties (Clarke et al., 2014). Ionophore coccidiostats have long been used in the management of livestock and poultry production worldwide as antibiotic feed additives for the control of coccidiosis and the improvement of feed conversion efficiency (Chapman, 2017; Noack et al., 2019; Blake et al., 2020). Monensin, lasalocid, salinomycin, narasin, maduramicin, and semduramicin are the 6 approved ionophore coccidiostats marketed as anticoccidial drugs or feed additives for use in the management poultry production within the EU (Commission Regulation (EC) No 1831/2003). Ionophore coccidiostats have demonstrated anticoccidial and antibacterial properties in poultry with a

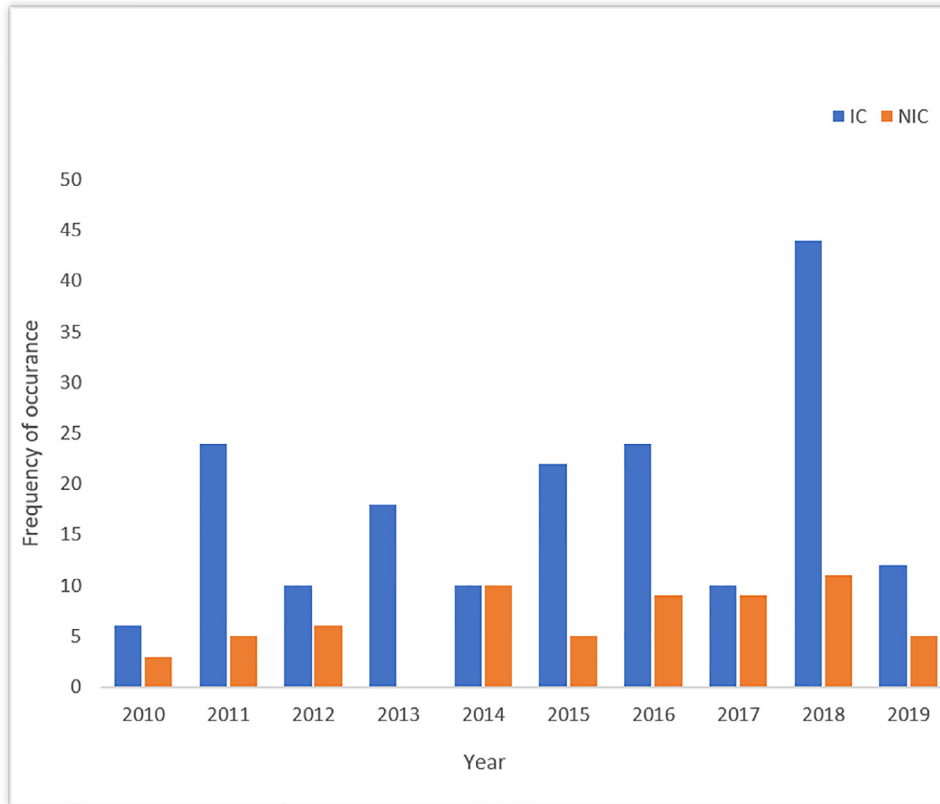


Figure 1. Bar chart depicting the EFSA yearly report (2010–2019) on the occurrences of noncompliant samples containing ionophore (IC) and non-ionophore (NIC) coccidiostats residues in commercial poultry eggs.

broad spectrum of activity against pathogenic *Eimeria* parasites (Anadón and Martínez-Larrañaga, 2014). This, together with the low risk of resistance development in coccidia contributed to the popularity of ionophores in poultry production worldwide (Anadón and Martínez-Larrañaga, 2014; Clarke et al., 2014). Unfortunately, the clinical value of these drugs is paralleled by the high potential for accumulation of their residues in eggs. As a result, they are not approved for use in laying hens (Olejnik et al., 2011; Piątkowska et al., 2012).

The mechanism of action of ionophore coccidiostats is based on the formation of complexes with sodium, potassium, magnesium, and calcium ions and subsequent disruption of the ionic balance across the cell membrane of the sporozoites and merozoites (Botsoglou and Fletouris, 2001; Chapman, 2007; Clarke et al., 2014). The disruption of normal ion exchange inside the cell and its subcellular organelles affects the control of physiological processes, resulting in the parasite's death (Botsoglou and Fletouris, 2001; Clarke et al., 2014). Ionophore coccidiostats differ greatly in terms of their chemical structure and affinity for specific cations which can be monovalent (sodium and potassium cations), forming neutrally charged ion complexes as seen for monensin, narasin, salinomycin, maduramicin, and semduramicin, or divalent (calcium and magnesium) as seen for lasalocid (Anadón and Martínez-Larrañaga, 2014). Unlike the synthetic chemical coccidiostats that act intracellularly against *Eimeria* parasites, ionophore coccidiostats exert their anticoccidial activity against the extracellular stage of the parasite development present within the

lumen of the intestine and, therefore, prevent the disease at the stage of invasion of the intestinal cells (Chapman, 2007, 2017; Clarke et al., 2014). Furthermore, ionophore coccidiostats inhibit *Eimeria* parasite reproduction and multiplication but do not completely eliminate it from the host intestinal tract, thereby preventing the manifestation of clinical coccidiosis (Chapman, 2017). Conversely, despite their role in controlling coccidiosis in poultry, ionophore agents have a narrow margin of safety in both the target and non-target animal species (Dowling, 1992). The lowest toxic dosage level of exposure to ionophore coccidiostats at which the manifestation of adverse toxic effects may occur in chickens are 125 to 150 mg/kg for lasalocid, 121 to 150 mg/kg for monensin, 100 mg/kg for narasin and 100 mg/kg for salinomycin (Dowling, 1992). Generally, intoxication and the manifestation of the first clinical signs of toxicity may occur when 20 to 50% of the prescribed safe dosage levels of the administered drugs were exceeded (Dowling, 1992). This may occur due to accidental overdose or sometimes due to uneven distribution of the drug throughout the feed after mixing (Dowling, 1992).

SOURCES OF EXPOSURE IN LAYING HENS AND INCIDENCE OF OCCURRENCE IN EGGS

Feed cross-contamination is a well-known issue that may arise due to a variety of circumstances such as human error, production processes, and handling

procedures at the feed mill, during transportation, and at the farm (Kennedy et al., 1996; O'Mahony et al., 2012; Dorne et al., 2013; Rokka et al., 2013; Spiegel et al., 2013; Roila et al., 2019). Several studies have reported that feed cross-contamination with a carryover of ionophore coccidiostats has been identified as the most common source of exposure in laying hens (Kennedy et al., 1996; Borràs et al., 2011; Rokka et al., 2013; Spiegel et al., 2013; Roudaut and Fournet, 2017; Roila et al., 2019). It is widely accepted that under practical conditions in feed mills during the production of medicated and non-medicated feeds in the same line of production, some traces of the medicated feed batch may remain in the production line, and this carryover can contaminate subsequent batches of non-medicated feed (Kennedy et al., 1996; Borràs et al., 2011; O'Mahony et al., 2012; Annunziata et al., 2017). The risk and degree of cross-contamination depend on the equipment and methods used in the feed mill as well as the electrostatic properties of the premix formulation (HAFEZ, 1991; Kennedy et al., 1996). This is because additives in powdered forms are more difficult to flush from equipment between batches than those in granular form (HAFEZ, 1991; Kennedy et al., 1996). As a result of these concerns and to minimize carry-over of ionophore coccidiostat into non-medicated feed, the EU has laid down procedures for the manufacture, placing on the market, and use of medicated feed (Commission Regulation (EC) No 4/2019). The regulation obliged animal feed manufacturers that are using coccidiostats in the same line of production of non-medicated feed to make great efforts to prevent cross-contamination of non-medicated feed. As cross-contamination of non-medicated feed was found to be inevitable and may happen during feed manufacturing, transportation, or storage, such feed must be safe for both the animal receiving it and the consumers of its edible food products (Olejnik et al., 2014). As a result, the EU under (Commission Regulation EU No 574/2011, 2011) has established a threshold of 1% carryover of the maximum authorized dosage as acceptable in non-medicated feed intended for laying hens (Olejnik et al., 2014; Annunziata et al., 2017; Roila et al., 2019).

In addition to unavoidable cross-contamination of feed, another potential source of exposure for laying hens is the contaminated environment when hens are housed in a deep litter system (HAFEZ, 1991; Cannavan and Kennedy, 2000). This may result from a failure to properly clean contaminated pens that were used to rear pullets before their transition to laying phase and such can cause the recycling of drugs in birds through fecal and dust contamination (HAFEZ, 1991; Cannavan and Kennedy, 2000). A study was conducted to explore the fecal recycling of coccidiostats residues in laying hens; residues depletion was found to be longer in birds placed on deep litter systems than in the birds kept in cages (HAFEZ, 1991; Kennedy et al., 1996; Cannavan and Kennedy, 2000). This discrepancy was thought to be related to the recycling of coccidiostats by ingestion of contaminated feces, which contributes significantly to the accumulation

and persistence of residues in eggs (HAFEZ, 1991; Kennedy et al., 1996; Cannavan and Kennedy, 2000).

Over the past decade, an increase in consumer-driven trends toward cage-free eggs has been fueled by the societal concern for food animal welfare (Kollenda et al., 2020). As a result, numerous recent efforts have been made by animal welfare organizations working under the umbrella of the European Citizens Initiative "End the Cage Age" which includes members from all around the EU (Kollenda et al., 2020; Poultry World, 2021). The EU parliament has responded to the call and set a plan to phase out the cage system by the year 2027 (Poultry World, 2021). The introduced cage-free system of production, in which laying hens are managed on litter systems such as aviaries, may probably contribute to the higher risk of disease outbreaks on poultry farms (e.g., coccidiosis) (Gauly, 2006; Blake et al., 2020). As a result, it may be possible that shortly after the complete transition to a cage-free system, controlling coccidiosis outbreaks would become one of the major issues that cage-free egg producers would have to face (Martina The Poultry Site, 2018; Kollenda et al., 2020). This could lead to situations where some farmers may unlawfully dose laying hens with ionophore coccidiostats for combating coccidiosis. When compared to the enriched cage system of egg production, the cage-free method is likely to be linked with greater production risks such as rapid disease transmission within the flock and more degraded eggs, as well as high management costs that lay the weight of higher egg prices on consumers (Gauly, 2006; Kollenda et al., 2020; Trejo-Pech and Thompson, 2021).

The incidence of ionophore coccidiostats residues in eggs has been well documented in the literature for the past two decades, and the majority of the reported results show that the presence of these feed additives in eggs is most likely the result of unavoidable carry-over of the feed additives to non-medicated feed intended for laying hens (Kennedy et al., 1998; Mortier et al., 2005; Olejnik et al., 2011; O'Mahony et al., 2012; Roudaut and Fournet, 2017). Based on the result of the yearly reports on the monitoring of veterinary medicinal products residues and other substances in live animals and animal products conducted by the EFSA from 2010 to 2019 across the EU, the number of non-compliant results for targeted and suspected egg samples obtained for registered coccidiostats revealed that ionophore coccidiostats were the most frequent coccidiostat feed additives found to contaminate laying eggs (Figure 1; EFSA, 2012, EFSA, 2013, EFSA, 2014, EFSA, 2015, EFSA, 2016, EFSA, 2017, EFSA, 2018, EFSA, 2019, EFSA, 2020, EFSA, 2021). Among the authorized ionophore coccidiostats, lasalocid was consistently detected as the most common coccidiostat residue in eggs (Mortier et al., 2005; Olejnik et al., 2011; Piątkowska et al., 2012; Roila et al., 2021). This is supported by the result of the yearly report of EFSA from 2010 to 2019 on the monitoring of veterinary medicinal products residues and other substances in live animals and animal products where lasalocid is characterized by a fluctuating yearly

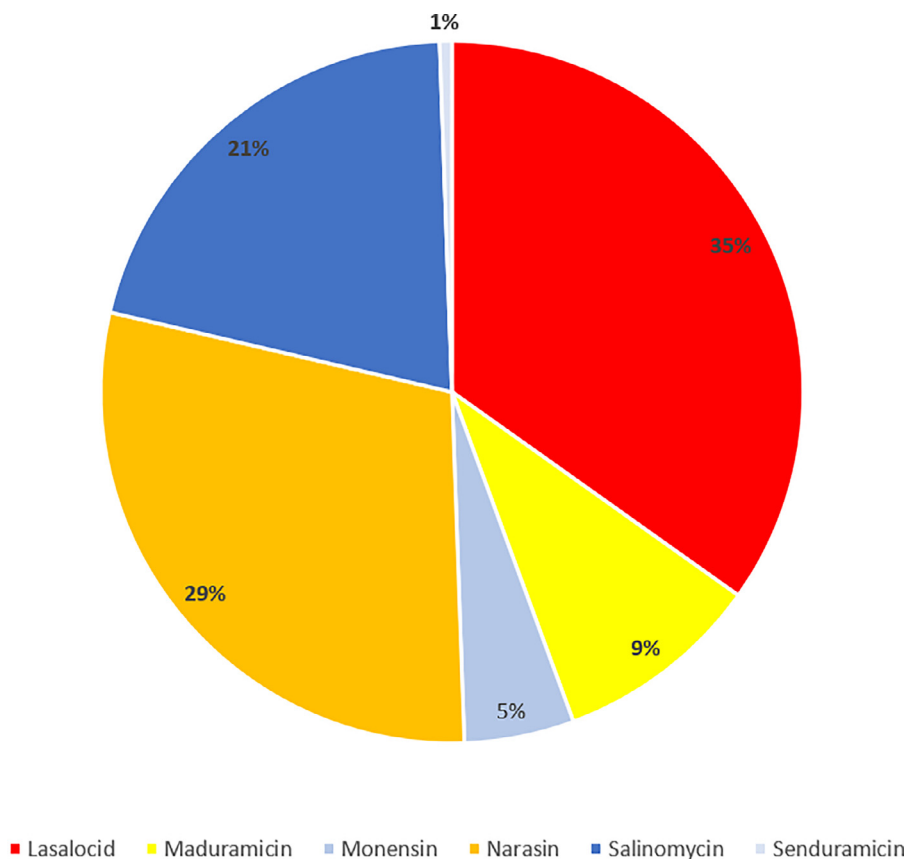


Figure 2. Pie chart depicting the EFSA yearly report (2010–2019) on the occurrences of noncompliant samples containing ionophore coccidiostats residues in commercial poultry eggs.

incidence of occurrence along the time frame considered and it appeared to have the highest frequency of occurrence in the non-compliant samples of commercial poultry eggs as shown in Figure 2 (EFSA, 2012, EFSA, 2013, EFSA, 2014, EFSA, 2015, EFSA, 2016, EFSA, 2017, EFSA, 2018, EFSA, 2019, EFSA, 2020, EFSA, 2021). To ensure a proper function of international trade and to safeguard public health, the EU under Regulation (Commission Regulation EU No 610/2012, 2012) has established a maximum level (ML) for all the approved ionophore coccidiostats in poultry eggs ($2 \mu\text{g}/\text{kg}$ for monensin, maduramicin, narasin, and senduramicin and $3 \mu\text{g}/\text{kg}$ for salinomycin) that resulted from unavoidable carry-over of these substances in non-target feed except for lasalocid (Piątkowska et al., 2012; Roudaut and Fournet, 2017). In contrast, based on EU Regulation (Commission Regulation EU No 610/2012, 2012) on pharmacologically active substances and their classification regarding MRL in food of animal origin, lasalocid was the only registered ionophore coccidiostat with an established maximum MRL of $150 \mu\text{g}/\text{kg}$ in poultry eggs (Piątkowska et al., 2012; Roudaut and Fournet, 2017). The presence of residue levels of ionophore coccidiostats in eggs exceeding the established ML or MRL is most likely not due to cross-contamination of feed, but rather to a non-authorized usage of these agents in laying hens, hence resulting in the production of residue contaminated eggs to the consumer (EFSA, 2007a). Therefore, as people become more aware of the importance of food safety and quality standard, it is

becoming increasingly important for poultry feed manufacturers and egg suppliers to strictly adhere to the food safety regulations established by the EU for supplying safe and wholesome products for human consumption. For this reason, pharmacokinetic studies have usually been employed to provide the basis for defining residue depletion profiles of a drug and the legally acceptable level (MRL) for edible tissues of animal origin which can be used in evaluating consumer risk of exposure (Donoghue, 2005; Dorne et al., 2013). Withdrawal period is the time needed to eliminate the drug below a given threshold after cessation of administration (Marmulak et al., 2010). Observance of these periods for drugs ensures that the risk of exceeding their specific MRL is managed.

PHARMACOKINETICS

In order to determine how residues of administered drugs are eliminated in food-producing animals, specific pharmacokinetic studies need to be carried out (Baggot, 1992). This approach allows for measuring the processes that govern drug movement across the body, from absorption and distribution to metabolism and excretion which can be modeled using different mathematical methods to estimate important pharmacokinetic parameters (i.e., bioavailability, volume of distribution, clearance, and elimination half-life; Baggot, 1992; Toutain and Bousquet-Mélou, 2004a,b,c,d).

Absorption

Following oral exposure of laying hens to ionophore coccidiostats, drugs are rapidly absorbed from the lumen of the gastrointestinal tract into the enterocytes, and subsequently transported to the liver through the hepatic portal vein before reaching the systemic circulation (Atef et al., 1993a,b). Because laying hens typically get exposed to ionophore coccidiostats via the oral route, bioavailability, which determines the fraction of the administered dose of the drug that reaches the systemic circulation, is an important consideration (Botsoglou and Fletouris, 2001). Unlike salinomycin having a high oral bioavailability of about 73% (Atef et al., 1993b), a relatively low bioavailability (>30%) was reported for monensin (Goetting et al., 2011; Henri et al., 2012; Dorne et al., 2013). There are no bioavailability data available that demonstrate the extent of absorption of lasalocid, narasin, maduramicin, and semduramicin in chickens or laying hens. However, they were reported to be rapidly absorbed from the digestive tract (Goetting et al., 2011; Anadón and Martínez-Larrañaga, 2014). Furthermore, nothing is known about the effect of the efflux pumps that are present in the liver and at the luminal surface of the intestinal wall on the oral bioavailability of ionophore coccidiostats.

Distribution

Following oral absorption, ionophore coccidiostats are widely distributed, mainly in the extravascular space, and are being detected in all tissues including the liver, muscles, skin, fat, ovaries, and oviductal tissues (EFSA, 2007a,b, 2008a,b,c,d; Goetting et al., 2011; Dorne et al., 2013). Drug-related features like physicochemical properties and plasma protein binding, as well as animal-related characteristics such as cardiac output, and regional blood flows may all influence drug distribution throughout the body (Botsoglou and Fletouris, 2001). In chickens, ionophore coccidiostats were shown not to bind extensively to plasma proteins. Under *in vitro* conditions, the plasma protein binding capacity of monensin and salinomycin was estimated at below 30% (Atef et al., 1993a,b). No data is available for the majority of ionophores in laying hens. Because of their high hydrophobicity and low protein binding, ionophore coccidiostats diffuse throughout the body and attain higher concentrations in tissues than in plasma. This is typically reflected in the high values of their volume of distribution (Atef et al., 1993a; Henri et al., 2012).

Metabolism

In poultry, ionophore coccidiostats are extensively metabolized, primarily in the liver (EFSA, 2007a,b, 2008a,b,c,d) by the cytochrome P-450 superfamily (Catherman et al., 1991; Henri et al., 2008; Russell, 2021). Phase I metabolism that involves oxidative processes of either single or combined hydroxylation and O-demethylation constitute the main biotransformation

pathways for these molecules (EFSA, 2007a,b; 2008a,b, c,d). Little is known about the involvement of Phase II processes in ionophore coccidiostats metabolism. The unchanged parent molecule appears to be the major residue and is considered a marker residue in the liver and target tissues (EFSA, 2007a,b, 2008a,b,c,d). For most ionophore coccidiostats, a great number of metabolites of increasing polarity have been detected in the excreta based on radioactivity studies. These metabolites seem to pose a minor concern as they constitute a small percent fraction of not more than 10% of the total radioactivity of the parent molecule and the identity of some of them have not yet been identified (EFSA, 2007a,b, 2008a,b,c,d).

Excretion

Pharmacokinetic studies in poultry carried out with radio-labeled compounds have shown that ionophore coccidiostats and/or their metabolites are rapidly eliminated from the systemic circulation (EFSA, 2007a,b, 2008a,b,c,d). Avian species are classified as good biliary excretors and, interestingly, ionophore coccidiostats are predominantly excreted through the bile in feces, with only a slight fraction of the drug and/or metabolites passing through the urine (Botsoglou and Fletouris, 2001; Goetting et al., 2011). Similarly, it is possible to have a long elimination half-life when entero-hepatic recirculation of the drug occurs or as a result of ingestion of droppings (HAFEZ, 1991). In addition to the hepatobiliary and renal route, egg-laying is considered to be another route of drug excretion in laying hens, particularly efficient for highly lipophilic drugs like most ionophores. This is part of the unique avian physiology and the ability to accumulate drug residue in developing egg compartments without redistributing it back to the systemic circulation until oviposition (Donoghue et al., 1996; Donoghue, 2005). For this reason, it is thought that hens that have a low egg production rate or skip more days for egg-laying would take a longer time to eliminate residues accumulated in the follicular yolk (Donoghue et al., 1996).

PHYSIOLOGY OF EGG FORMATION AS A DETERMINANT OF RESIDUES ACCUMULATION

Understanding the physiology of oviposition is essential to understanding how drug residues accumulate in eggs. The physiology of egg formation in laying hen is well explained (Johnson and Woods, 2007; Nys and Guyot, 2011; Réhault-Godbert and Guyot, 2018; Sah and Mishra, 2018) and can be briefly summarized in this context as depicted in Figure 3. An egg is a biological matrix comprised of 2 distinct components, the egg white (albumen) which constitutes about 60% of the total egg weight and predominantly consists of water (88%) while egg yolk, being a mixture of lipoproteins and other plasma components, constitutes about 30 to

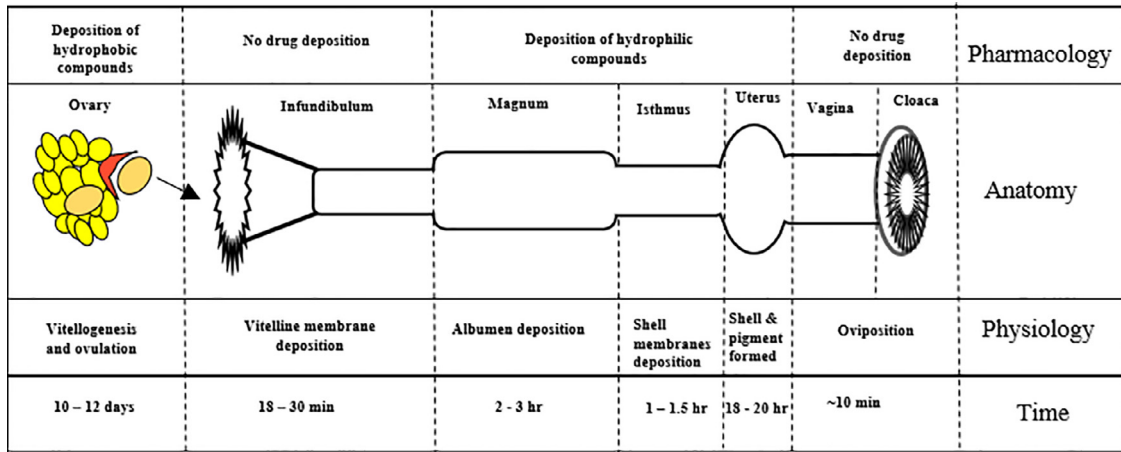


Figure 3. Diagram depicting the interplay between the physiology of egg formation and residue transfer and deposition in eggs.

33% of the total egg weight and consists of approximately 48% water (Nys and Guyot, 2011; Van Der Fels-Klerx et al., 2017). The eggshell forms the outer layer which accounts for 9 to 12% of the total egg weight having calcium carbonate as 99% of its main constituents (Nys and Guyot, 2011; Van Der Fels-Klerx et al., 2017). Under normal conditions, a healthy laying hen can produce an egg approximately every 24 h, however, breed/strain-related differences exist (Tůmová et al., 2017). Egg formation occurs within 2 distinct anatomical structures of the hen's reproductive tract: the ovary and the oviduct (Réhault-Godbert and Guyot, 2018; Sah and Mishra, 2018). The ovary consists of a cluster of follicles that are in various stages of development (Réhault-Godbert and Guyot, 2018; Sah and Mishra, 2018). It secretes estrogen and other reproductive hormones which influence follicular growth together with the oviduct setting the stage for egg formation (Johnson and Woods, 2007; Nys and Guyot, 2011). In response to high levels of circulating estrogen, the liver synthesizes a large number of lipoproteins, which are yolk precursors that begin to deposit continuously in a concentric form in a single growing follicle until the time of ovulation (Johnson and Woods, 2007; Réhault-Godbert and Guyot, 2018). Receptor-mediated endocytosis seems to be the main transport mechanism of lipoprotein components from the blood into the yolk (Johnson and Woods, 2007; Réhault-Godbert and Guyot, 2018). It takes around 10 to 12 d for complete maturation of yolk in the ovary before ovulation will occur (Nys and Guyot, 2011). Unlike the ovarian follicle, which concentrates plasma lipoproteins to form egg yolk, the specialized cells of the oviduct synthesize and secrete proteins that will constitute the albumen (Nys and Guyot, 2011; Réhault-Godbert and Guyot, 2018). Before ovulation, they are stored in the tubular gland cells of the oviduct. After ovulation, the developed yolk is caught into the infundibulum and transversed through various segments of the oviducts (Nys and Guyot, 2011; Réhault-Godbert and Guyot, 2018). In the magnum, a highly water-soluble albumen protein is secreted into the lumen that surrounds the yolk forming a thick and thin layer of proteingel over a period of about 2 to 3 h after ovulation (Nys and Guyot, 2011;

Sah and Mishra, 2018). In the distal parts of the oviduct (isthmus and uterus), the albumen takes up electrolytes and water. This process is called "plumping". The time frame that involves the formation of the vitelline membrane, egg albumen, and eggshell membrane may last around 5 to 6 h (Nys and Guyot, 2011; Sah and Mishra, 2018). Moreover, as the yolk and the surrounding albumen continue to move by peristaltic action down through the isthmus to reach the shell gland (uterus), water and salts are added and the shell gland deposits calcium carbonate in a protein matrix to produce the characteristic rigid shell of a strain-specific color (Nys and Guyot, 2011; Réhault-Godbert and Guyot, 2018; Sah and Mishra, 2018). Overall, it takes about 18 to 20 h for the egg shell to be formed in the uterus before being laid. The complete process of egg formation to oviposition may take around 11 to 13 d (Nys and Guyot, 2011; Réhault-Godbert and Guyot, 2018; Sah and Mishra, 2018).

TRANSFER OF RESIDUES INTO EGGS AND DISTRIBUTION BETWEEN THE EGG WHITE AND YOLK COMPARTMENTS

Following oral exposure in laying hens, ionophore coccidiostats are intended to act in the gastrointestinal lumen, but they are absorbed systemically due to their lipophilic properties, which allow them to interact with and pass through the cell membranes of the intestinal tract (Chapman, 2007). This property is not essential for their parasiticidal activity but may contribute to residue formation in tissues (Kan and Petz, 2000). Upon reaching the bloodstream, they are widely distributed throughout the body including the ovary with growing follicles and the oviduct, where the egg white is formed and secreted (Kan and Petz, 2000). Some fraction of the circulating drug in plasma may become tightly bound to the lipoprotein yolk precursors produced in liver and transported to the ovary with subsequent incorporation into the developing yolk follicle through the daily layering of yolk material in a form of overlaying spheres (Donoghue et al., 1997; Kan and Petz, 2000).

Furthermore, the fraction of the circulating drug that is unbound to lipoproteins reaches the oviduct through the blood supply to the organ. Depending on the protein binding capacity of the drug, it may be bound to the albumen proteins synthesized and secreted by the oviductal glands during the deposition of egg white on the ovulated egg yolk while passing through the oviductal tract (Botsoglou and Fletouris, 2001; Furusawa, 2001). It was also observed that drug may be passively diffused into the egg white during the plumping phase when excess water is added to the egg white proteins (Furusawa, 2001).

Residues of administered drugs may be formed in developing eggs and retained there for days or weeks until eggs are laid (Kan and Petz, 2000; Goetting et al., 2011). Various efforts have been made to predict the preferred deposition of chemical residues in either the egg white or the yolk, which are the 2 major egg compartments to be considered when evaluating drug residues after exposure (Donoghue, 2001; Kan, 2003). It is known that egg white differs greatly from egg yolk in its chemical composition and that many veterinary drugs and feed additives employed in poultry production are preferably deposited in one of these compartments (Donoghue, 2005). The residue profiles of administered drugs that appeared in either egg white or egg yolk were often found to be very different (Kan and Petz, 2000; Furusawa, 2001; Goetting et al., 2011). Several studies have attempted to explain the critical factors that may influence the transfer of residue and model its distribution to either egg yolk or egg white compartment (Donoghue et al., 1997; Donoghue, 2001, 2005; Furusawa, 2001; Goetting et al., 2011; Hekman and Schefferlie, 2011; Schefferlie and Hekman, 2016).

It was observed that following drug exposure, residues may first appear in the egg white which seems to reflect the immediate plasma drug concentration since the total egg white content in an egg is formed within 3 h following ovulation (Kan, 2003). On the other hand, residue in egg yolk appears as late as 24 h post-exposure as a reflection of its daily deposition into the rapidly growing follicles until ovulation occurs (Kan, 2003). Therefore, it takes about 2 to 3 d and 8 to 10 d for a drug residue to reach a constant level in the egg white and yolk compartment, respectively (Kan, 2003). This is believed to be due to differences in the timeframe of the physiological processes that govern the formation of egg compartments (Kan and Petz, 2000). Apart from the drug physicochemical characteristics, the binding capacity to plasma proteins is also considered essential for the specific distribution in egg compartments (Botsoglou and Fletouris, 2001; Kan, 2003; Goetting et al., 2011). It is commonly recognized that high affinity of certain lipophilic compounds for egg yolk rather than albumen is related to the high lipoprotein content in the yolk matrix (Donoghue, 2001; Furusawa, 2001). Goetting et al. (2011) conducted a review of the literature on the pharmacokinetics of different veterinary medicinal products and their deposition in eggs. They concluded that physicochemical properties of a molecule alone cannot always

be used to predict its pharmacokinetics. Likewise, Schefferlie and Hekman (2016) suggested that lipid solubility alone is not the only chemical factor to influence the transfer rate and distribution of residues between the egg white and yolk compartments. Even when all of the aforementioned physicochemical parameters are taken into account, it is difficult to predict the distribution of residue between the egg white and yolk after exposure since some of the factors that determine the preference of distribution of the residues between the 2 compartments are still to be characterized (Furusawa, 2001; Kan, 2003; Donoghue, 2005; Schefferlie and Hekman, 2016).

Several studies have demonstrated ionophore coccidiostats transfer to specific egg compartments following feeding trials with diets containing ionophores at various cross-contamination levels (Akhtar et al., 1996; Kennedy et al., 1996; Kan and Petz, 2000; Rokka et al., 2005; Bodi et al., 2012; Vandenberghe et al., 2012; Olejnik et al., 2014; Varenina et al., 2015). Despite the structural similarities among ionophore coccidiostats, there are apparent differences in the rate of residue transfer and affinity to egg white and yolk compartments (Vandenberghe et al., 2012). Studies have demonstrated that majority of the commonly used ionophore coccidiostats tend to accumulate predominantly in the egg yolk (Akhtar et al., 1996; Rokka et al., 2005; Goetting et al., 2011; Olejnik et al., 2014; Varenina et al., 2015), except for monensin which distributes more widely to egg white (Vandenberghe et al., 2012; Goetting et al., 2011; Kan, 2003). Among the 6 licensed ionophore coccidiostats, lasalocid has been reported to have the highest transfer affinity and accumulation potential in the egg yolk (Vandenberghe et al., 2012; Goetting et al., 2011; Rokka et al., 2005; Mortier et al., 2005). As a result, the relationships between 1) the physiology of egg formation, 2) the physicochemical characteristics of the molecule and drug dosage and 3) their related pharmacokinetic implications must be investigated to fully understand the mechanisms that determine drug residue profiles in both egg white and yolk matrices.

The ratio of concentrations in yolk to albumen observed in some trials during steady state varies across individual ionophore coccidiostats as presented in Table 1. Of note, because the concentration of the drug in albumen in certain trials (Rokka et al., 2005; Bodi et al., 2012) was below the limit of detection, it was not always possible to calculate this ratio.

Despite the fact that a significant set of empirical data has been collected on drug transfer to eggs, modeling these processes may pose a great challenge (Donoghue, 2005). Even though the classical pharmacokinetic models and principles are capable of describing the time-dependent course of residues in plasma and tissues, they fail to do so in developing egg compartments (Schefferlie and Hekman, 2016). Indeed, in contrast to drug concentrations in the egg white, its concentrations in egg yolk do not parallel with the concentrations in plasma (Schefferlie and Hekman, 2016). In the case of ionophore coccidiostats, some studies aimed to quantify the dynamics of

Table 1. Physicochemical properties, plasma protein binding (%) and concentration ratio in egg yolk to egg white of ionophore Coccidiostats authorized in the EU as feed additives after feeding trials experiments in laying hens (as reported by [Olejnik et al. 2014](#)).

S/N	Drug name	Molecular weight	LogP	pKa	Plasma Protein binding (%)	Dosage (mg kg ⁻¹) of feed	Duration of exposure (days)	Yolk/white conc. ratio	References
1	Lasalocid	612.77	6.547	3.15	–	12.5	14	197.3	Vandenberge et al. 2012
2	Monensin	787.00	6.591	7.9	–	12.5	14	0.7	Vandenberge et al. 2012
3	Narasin	934.16	4.337	4.01	–	70	7	3.2	Kan and Petz 2000
4	Salinomycin	692.85	3.716	4.26	22.8	0.15	13	18.5	Akhtar et al. 1996
5	Semduramicin	772.98	6.099	4.37	19.78	0.27	14	18.5	Olejnik et al. 2014
6	Maduramicin	895.06	3.505	5.39	–	0.005	21	–	Bordi et al. 2012
7	Narasin	934.16	4.337	4.01	–	2.5	21	–	Rokka et al. (2005)

residues transfer into the egg white and yolk compartments ([Bodi et al., 2012](#); [Vandenberge et al., 2012](#); [Olejnik et al., 2014](#); [Varenina et al., 2015](#)). All these authors used classical pharmacokinetic concepts and reported the concentration of the measured residues in eggs as such after drug administration. So far, all these attempts failed to combine the time-dependent changes in plasma drug concentrations that cause the resulting residues in egg white and yolk compartments.

FUTURE MODELING PERSPECTIVES FOR PREDICTING RESIDUE FORMATION IN EGGS

Major advances in information technology have made a significant impact on the development of modern computers and advanced analytical software packages that became available for use in mathematical modeling and simulation in the aspect of human and veterinary medicine and toxicology. Considering the physiology of egg formation and the mode of drug excretion from the body through egg-laying, a physiologically based pharmacokinetic (**PBPK**) model could be an alternative solution for modeling and simulating residue transfer to eggs in avian species. This is because PBPK modeling is a robust predictive tool that describes the absorption, distribution, metabolism, and excretion of xenobiotics in an organism based on anatomic parameters as well as physiological and biochemical mechanisms ([Baynes and Riviere, 2014](#); [Lin et al., 2016](#)). Because of the combination of physiological, biochemical, and physicochemical characteristics, the PBPK modeling approach has the potential to determine residue levels in multiple tissue compartments in food-producing animals after exposure via diverse routes and across various species ([Lautz et al., 2019](#)). This modeling tool also enables a direct correlation of tissue concentrations to specific targets like MRL, therapeutic ranges, or toxic concentrations following any type of exposure ([Baynes and Riviere, 2014](#); [Lautz et al., 2019](#)). Today, PBPK modeling is gaining acceptability from the global food safety regulatory authorities and is now established as a key and indispensable tool in the evaluation of risk assessments of toxicants ([Lin et al., 2016](#); [Lautz et al., 2019](#)). Unlike predicting residue concentrations in edible tissues of broilers, modeling residue concentrations in eggs is still a work in progress. The development of PBPK models in

poultry species has long been hampered by the lack of a comprehensive database for physiological, anatomical, and biochemical parameters in these species. Interestingly, [Wang et al. \(2021\)](#) have now published physiological parameter values for PBPK modeling in chickens and turkeys which they extracted from the existing literature. Concerning the prediction of residue concentration in eggs, [Lautz et al. \(2020\)](#) have developed a generic open-source PBPK model in chicken through the integration of meta-analyzed physiological parameters into an R-based algorithm. The model was calibrated and validated for 7 chemical substances that are relevant to food and feed safety, and predictions of plasma, tissue, and egg concentrations were made, which are in excellent agreement with published data ([Lautz et al., 2020](#)). Recently, [Enomoto et al. \(2021\)](#) have reported residues deposition and depletion in eggs following multiple oral administrations of trimethoprim-sulfamethoxazole in laying hens taking into account differences in the rate of excretion in egg white and yolk. These 2 references provided insight into the physiological prediction of drug residue in eggs. The critical relevance of this novel approach may be supported by the fact, that during the review process of the current manuscript, 2 new PBPK models for prediction of drug residues in birds have been published. The first one describes a web-based interactive PBPK model for predicting meloxicam residues in broilers and laying hens ([Yuan et al., 2022](#)). The model adequately simulated published pharmacokinetic data for plasma, tissue, and egg concentrations, and was able to predict withdrawal intervals following extra-label use of meloxicam in these birds. The second paper describes a generic avian PBPK model and its application in chickens, bobwhite quail and mallard ducks ([Baier et al., 2022](#)). It included an ovulation compartment which allowed predicting the concentration of chemicals in both egg white and yolk. The model was tested with nine chemicals for which experimental data was available. The overall precision of the estimates was described as “good”, but for some chemicals the precision was limited. Despite these new achievements, there is still a need to improve PBPK modeling of drug residues in this specific type of animal product. For this to happen, it is necessary to consider the interplay between the time events in the physiology of egg formation in laying hen and the mechanisms involved in xenobiotic transfer. Since most ionophore coccidiostats are hydrophobic and their half-life in eggs is relatively long ([Vandenberge](#)

et al., 2012; Varenina et al., 2015), it is possible to draw inspiration from models of transfer of persistent organic pollutants including age-dependent lipid dynamics in laying hens developed in environmental toxicology (Fournier et al., 2015). The growth of the laying hen and the sequences events during and after moulting episodes will have an impact on the dilution effect in the tissues and on the hydrophobic molecules that will accompany changes in lipid proportions (Fournier et al., 2015). During successive egg-laying, excretion of residue into the laid egg reduces its concentration in body circulation. Moreover, for appropriate modeling of residue formation in an egg yolk, it is necessary to collect additional physiological parameters associated with laying, such as the minimum follicular weight at the beginning of the commencement of the rapid growth phase (before the initial follicle enters the rapid growth phase) (Fournier et al., 2015), the concentration of lipids in the proportion of neutral lipids in a matured follicle ready for ovulation (Nys and Guyot, 2011), the maximum and minimum oviposition rate (at the period of increasing and declining phase of egg laying, respectively), and the persistence of oviposition (Grossman and Koops, 2001) and egg weight at maturation (Adams and Bell, 1998). In a modeling attempt, the duration of the rapid growth of each follicle can be assumed to be approximately 10 days. The initialization of such rapid growth for each follicle can occur in a staggered fashion (Waddington and Walker, 1988; Nys and Guyot, 2011). Additionally, a robust assessment of total body clearance is a key input parameter in any PBPK model. This is because renal and hepatic clearance of administered drugs cannot be readily segregated in vivo in birds, as such one may need to recourse to whole cell (primary chicken hepatocytes) or subcellular (liver microsomal or S9 fractions) assays. Similarly, plasma protein binding may be assessed in vitro. The data obtained in vitro may be used for quantitative in vitro-to-in vivo extrapolation (qIVIVE) with scaling factors for the laying hens (Girirajan et al., 2021).

CONCLUSIONS

This review highlighted the evidence that ionophore coccidiostats are the most widely used coccidiostat feed additives within the EU. Their carryover in the feed of non-target poultry species (laying hens) has been identified as the main reason of their occurrence in commercial poultry eggs. Among the 6 registered ionophore coccidiostats within the EU, lasalocid appears to be the most common contaminant of commercial poultry eggs. The physicochemical properties of individual compounds, the physiology of the laying hen and the biology of egg formation are believed to govern the residue transfer rate and its distribution between the egg white and yolk compartments. However, there is limited data on the pharmacokinetics and metabolism, plasma protein binding, and plasma/tissue partition coefficient of ionophore coccidiostats in laying hens. We, therefore, recommend

that comprehensive studies should be conducted to provide primary data for effective modeling of the residue formation for these feed additives in eggs. Since the classical pharmacokinetic approach did not allow predicting drug disposition into eggs with sufficient precision, it is believed that PBPK modeling will provide a better understanding of the factors that influence coccidiostat residue transfer into specific egg compartments. All this effort is needed to further improve the safety of this foodstuff.

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DISCLOSURES

The authors report there are no competing interests to declare.

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