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Mini review

Integrating adverse effects of triazole fungicides on reproduction and physiology of farmland birds

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The extensive use of pesticides has been recognized as one of the major factors negatively impacting birds in agricultural habitats. One of the pesticide groups most used worldwide are triazole fungicides due to their effectiveness in controlling phytopathogenic fungi in cereals, vineyards and orchards. In the last decades, different experimental studies have reported important negative effects on the health and fitness of birds after exposure to triazoles. Birds can be exposed throughout the year through different routes, including oral uptake, dermal contact with treated surfaces and inhalation by overspray. Yet, the ingestion of treated or sprayed material is the principal route. The most alarming effect of triazoles, which can even occur several months after cessation of the exposure, is the decreasing reproductive outputs of birds, including delay in the onset of laying dates, reduced clutch size and hatching rate, and increased mortality of chicks. In order to synthesize the data and knowledge about the toxic effects of triazoles at different levels of biological organization, here we propose an dverse outcome pathway (AOP) on the mechanisms by which triazoles can affect avian reproduction and physiology. The reported effects highlight that the current risk assessment needs some improvements to avoid undesired effects on birds, especially long-term effects that can influence stability and viability of avian populations from agricultural habitats.

Keywords: agricultural habitats, anthropic impact, breeding, pesticides, risk assessment

The extensive use of pesticides has been recognized as one of the major factors negatively impacting biodiversity in agricultural habitats (Moreau et al. 2022, Mohanty 2024), being the most studied type of pollutants affecting birds in the last 20 years (Richard et al. 2021). Their use aims to increase the productivity and profitability of crops, reducing their competitors (herbicides), consumers (insecticides) or diseases (fungicides). The most immediate effects of pesticides on wildlife are due to toxicity caused by direct exposure with acute effects (e.g. death) occurring after short-term

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exposures to high pesticide levels, or with chronic effects (e.g. affected reproduction) that can result from continuous or repeated exposures to lower doses (Moreau et al. 2022) as well as from short-term exposures at critically sensitive life stages of development or during reproduction (EFSA 2023).

The family of triazole fungicides is one of the pesticide groups whose use has spread worldwide since the 1970s–1980s, motivated by their effectiveness in controlling phytopathogenic fungi in vineyards, orchards or cereals, among other crops (Ribas e Ribas et al. 2016, Atwood and Paisley-Jones 2017). Like any other pesticide, the use of triazoles in agriculture is approved under the assumption that they have no deleterious effects on the environment. To evaluate this, substances must be subjected to a risk assessment before they can be approved for marketing, a process that in the European Union (EU) is established by the Regulation EC/1107/2009. The specific provisions for conducting an environmental risk assessment of pesticides for birds and mammals in the EU are established in the corresponding Guidance elaborated by the European Food Safety Authority (EFSA) (EFSA 2023). This Guidance determines that pesticides cannot be approved if they pose a risk to individual survival or reproduction, which are set as surrogate protection goals to preserve bird and mammal populations. Although the current Guidance is rather new, these surrogate protection goals were already formulated in the previous version of the Guidance, published in 2009 (EFSA 2009).

Like the majority of current-use pesticides, triazole fungicides do not seem to directly compromise avian survival under environmentally realistic exposure scenarios (Grote et al. 2008, Lopez-Antia et al. 2018, 2021). However, in the last 20 years, different experimental studies with birds have reported negative effects of triazoles on the health and fitness of birds (Supporting information). One of the most alarming impacts of triazoles is the decreasing reproductive output of birds, which occurs even if the exposure happens several months before reproduction. These effects include, among others, delay in the onset of laying dates (Fernández-Vizcaíno et al. 2020), reduced clutch size (Lopez-Antia et al. 2018) and hatching rate (Lopez-Antia et al. 2013, 2018, 2021), and increased mortality of chicks (Lopez-Antia et al. 2013, Ortiz-Santaliestra et al. 2020). These outcomes, reported under realistic scenarios of exposure to triazoles, highlight that the abovementioned protection goals set in avian risk assessment would not be met and that the current risk assessment would need some improvements to avoid undesired effects on birds (Robinson et al. 2020), especially those long-term effects that can influence population stability and viability (Bean et al. 2023). In this context, the European Commission Regulation 283/2013 setting out the data requirements for the risk assessment of pesticide active substances in the EU, establishes that long-term toxicity of pesticides to birds shall not be evaluated if exposure during the breeding season is unlikely to happen. Although the Regulation also sets that long-term toxicity will be regarded if delayed effects occur during the breeding season, the standard reproductive toxicity tests recommended by EFSA (2023) to characterize long-term effects of pesticides on birds (i.e. OECD 1984, USEPA 2012) do not contemplate any scenario in which reproductive effects happen, as reported in the above-mentioned studies, with a delay of several months.

To understand the reasons why triazoles affect avian reproduction, it is necessary to elucidate the mechanisms of toxicity of these fungicides at the sub-organism level. This level of effects is commonly overlooked in environmental risk assessments, given the difficulties of linking those effects to responses at the apical (e.g. individual mortality) or ecological (e.g. population declines) levels (Rattner et al. 2023). A way to synthesize the data and knowledge about the toxic effects of chemicals at different levels of biological organization, which can be useful to both risk assessors and researchers in linking sub-organismal to apical effects, is through the development of an adverse outcome pathway (AOP) (Ankley et al. 2010). An AOP is designed to connect a molecular initiating event (MIE) as a result of the interaction of a chemical with a biomolecule, through a sequence of key events (KE) at different levels of biological organization (cellular, tissue, organ, organism and population), to one or several adverse outcomes (AO), which are effects occurring at individual or population levels (Willett 2014, Rattner et al. 2023). The increasing concern regarding the effects of triazoles on the reproduction of wildlife species, including birds, has triggered the development of research focused on elucidating the mechanisms of action behind these responses.

The objective of this review is to explore the published information on the mechanisms by which triazoles can affect avian reproduction to propose an AOP based on those mechanisms, completed as much as possible with studies conducted in birds and supported when needed by information generated from other species or in vitro (Fig. 1). In addition, we summarize other effects observed in birds exposed to triazoles that, even if not necessarily linked to the triazole mode of action, might also be linked to reproductive effects.

Triazoles: use, environmental fate and bird exposure

Phytopathogenic fungi can affect seeds during sowing and leaves and fruits during development in crops like cereals, vineyards and orchards (Cook et al. 1999, Shahinasi et al. 2017). To prevent this, triazoles are either sprayed on plants (spring–summer) or applied as seed coating treatments during sowing (autumn–winter). Thus, birds can be exposed to triazoles during different seasons throughout the year, and exposure may happen through different routes, the main one being the ingestion of treated or sprayed material such as plants, insects, water or seeds (EFSA 2009). Applying the pesticide directly into the seeds (coating seeds), instead of spraying it, reduces the quantity of product that is released to the environment as the pesticides are placed exactly where their action is needed, also reducing the cost of the treatment and the exposure of some non-target organisms (Sohail et al. 2022). These seeds should be buried during sowing to reduce

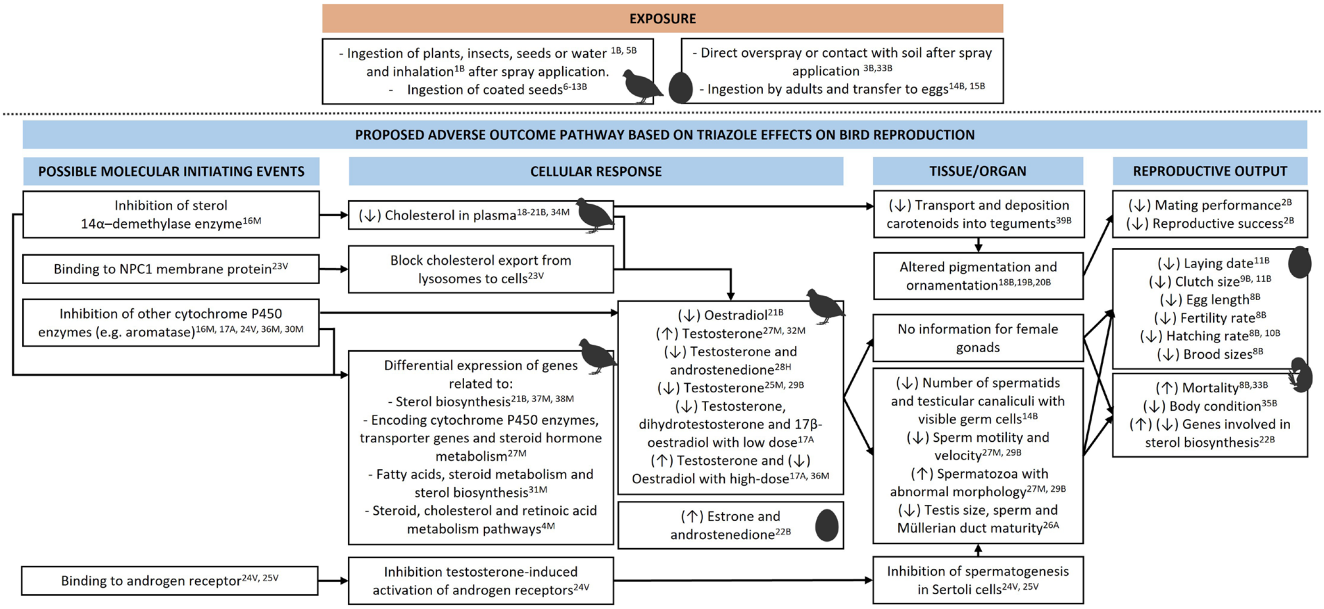

Figure 1. Ways of exposure of birds and eggs and the proposed adverse outcome pathway based on triazoles effects on bird reproduction. The black arrows represent suggested key event relationships; however, not in all cases these relationships have been proven as part of the same assay. References letter means M = Mammals; A = Amphibians; B = Birds; H = Humans; F = Fish; V = Vitro. List of references used: (1) Angelier et al. 2023; (2) Alonso-Alvarez et al. 2012; (3) Bro et al. 2016; (4) Robinson et al. 2012; (5) EFSA 2009; (6) Mateo et al. 2016; (7) Lopez-Antia et al. 2014; (8) Lopez-Antia et al. 2016; (9) Fernández-Vizcaíno et al. 2022; (10) Fernández-Vizcaíno et al. 2023; (11) Millot et al. 2015; (12) de Montaigu and Goulson 2022; (13) Esther et al. 2022; (14) Grote et al. 2008; (15) Bellot et al. 2022a, b; (16) Zarn et al. 2003; (17) Poulsen et al. 2015; (18) López-Antía et al. 2013; (19) López-Antía et al. 2018; (20) López-Antía et al. 2021; (21) Fernández-Vizcaíno et al. 2020; (22) Fernández-Vizcaíno et al. 2024; (23) Trinh et al. 2017; (24) Lv et al. 2017; (25) Roelofs et al. 2014; (26) Svanholm et al. 2021; (27) Tully et al. 2006; (28) Machado et al. 2021; (29) Serra et al. 2023; (30) Munkboel et al. 2019; (31) Goetz and Dix 2009; (32) Goetz et al. 2007; (33) Ortiz-Santaliestra et al. 2020; (34) Peffer et al. 2007; (35) Gaffard et al. 2022; (36) Chen et al. 2019; (37) Dimopoulu et al. 2017a; (38) Dimopoulu et al. 2017b; (39) McGraw and Parker 2006.

their availability for granivorous wildlife, and even although some birds can look for buried seeds, an adequate sowing depth minimizes the exposure chances also for those species. Nevertheless, because of spills or unprecise sowing, there are always some treated seeds that remain on the field surface, where they are available to wildlife species (De Snoo and Luttik 2004). The consumption of treated seeds can drive exposure to large amounts of pesticide within a short period (Prosser and Hart 2005, Lopez-Antia et al. 2016, de Montaigu and Goulson 2022, Fernández-Vizcaíno et al. 2023) and has been reported in several large granivorous bird species like red-breasted geese *Branta ruficollis* (Mateo et al. 2016), red-legged partridges *Alectoris rufa* (Lopez-Antia et al. 2014, 2016, Fernández-Vizcaíno et al. 2022, 2023), grey partridges *Perdix perdix* (Millot et al. 2015), carrion crows *Corvus corone*, rooks *Corvus frugilegus* or woodpigeons *Columba palumbus* (de Montaigu and Goulson 2022), and small granivorous passerines like skylarks *Alauda arvensis* (Esther et al. 2022), common chaffinch *Fringilla coelebs*, common starlings *Sturnus vulgaris* or house sparrows *Passer domesticus* (de Montaigu and Goulson 2022), among others.

Foliar application of triazole containing products is another potential source for avian exposure (Angelier et al. 2023). Apart from ingesting sprayed products, these foliar applications can result in dermal exposure because of bird contact with treated surfaces, especially soils. Standard soil half-life (DT$_{50}$, measured in reference soil at 20°C in the laboratory) of the triazoles currently approved for use as agricultural fungicides in the EU ranges from the moderate persistence of tetraconazole (DT$_{50}$ = 83.8 days) to the high persistence of tebuconazole (DT$_{50}$ = 365 days); only prothioconazole, which is quickly transformed into its desthio and S-methyl metabolites (EFSA 2007), shows no persistence, with a standard soil DT$_{50}$ of 0.44 days. Regarding sorption, organic-carbon normalized Freundlich distribution coefficient (K$_{foc}$) values reflect a slight mobility in all cases, varying from 570 ml g^{-1} of triticonazole (EFSA 2005) to 3522 ml g^{-1} of difenoconazole (EFSA 2011). This generally high persistence of triazoles in soils, although modulated by soil composition, environmental conditions (e.g. rainfall, sunlight incidence, wind) or biodegradation by soil biota, accounts for increased risk of dermal exposure of birds following spray applications. Dermal exposure of birds to pesticides is considered in the regulatory risk assessment as a minor route compared to oral uptake. However, several studies have pointed that contact with surfaces treated with pesticides could result in relevant toxicity to birds, and that risk assessments based on dietary exposure only could be flawed for certain substances (reviewed by Mineau 2011), although no studies in this context are available for triazoles. A particular way of contact exposure is that of eggs. Avian eggs can be exposed to triazoles if ground-nesting birds are breeding at the moment of fumigation (Bro and Clobert 2000, Casas et al. 2009). In this scenario, eggs can be either directly exposed to sprayed pesticides or considering the long-term persistence of many triazoles in soil (see above), laid on the surface of treated crops after pesticide applications (Bro et al. 2015, 2016, Ortiz-Santaliestra et al. 2020). A final exposure way for adult birds could be inhalation; however, this exposure route does not seem too relevant for birds in the case of triazoles and is also disregarded from avian risk assessment protocols (EFSA 2023). Triazole fungicides currently approved as agricultural pesticides in the EU are little volatile (the highest vapor pressure at 20°C, corresponding to penconazole, is as low as 0.336 mPa; EFSA 2008), which strongly limits the chances for vapour inhalation.

Triazole fungicide residues have been measured in samples collected from wild birds. For instance, tebuconazole was detected in faeces from wild partridges (range 5.09–38.61 ng g^{-1} wet weight), which was linked to coated seed consumption (Fernández-Vizcaíno et al. 2023), whereas high plasma levels of difenoconazole (10–156.7 pg g^{-1}), fenbuconazole (33.3–945 pg g^{-1}), tebuconazole (26–521.8 pg g^{-1}) and tetraconazole (24.3–8741.4 pg g^{-1}), were detected in blackbirds from a vineyard after fungicide overspray (Angelier et al. 2023). Despite these findings, triazoles used in current agriculture have not been identified as bioaccumulative. With the exception of prothioconazole, with an octanol–water partition coefficient (K_{ow}) of 2.00, they all show high lipophilicity, with K_{ow} values ranging from 3.24 for bromuconazole (EFSA 2010) to 4.36 for difenoconazole (EFSA 2011). Lipophilicity is commonly related to increased bioaccumulation potential; however, triazoles have been observed to be quickly metabolised and excreted by birds (Gross et al. 2020). For instance, Fernández-Vizcaino et al. (2023) reported that flutriafol was present as parent compound in 28% of the faecal samples of red-legged partridges feeding exclusively on treated seeds, and residues of that substance, as well as of tebuconazole and prothioconazole, disappeared from excreta quickly after the ingestion of treated food was terminated. Nevertheless, these studies do not cover the whole variety of triazoles used as agricultural fungicides and, despite some generalities like the high lipophilicity can be observed, there are differences among substances in physic-chemical properties that could determine different patterns of environmental fate and toxicokinetic.

Adverse outcome pathway based on triazole effects on bird reproduction

In Fig. 1 we present ways of exposure and propose an AOP based on triazole effects on bird reproduction. AOPs are not related to specific chemicals, so we cannot strictly talk about an AOP of triazoles. However, the development of AOPs is triggered by specific modes of action (i.e. interactions of toxicants with biomolecules) that in the AOP framework constitute the MIE. Here, the MIE corresponds to the mode of action of triazole fungicides. We propose this AOP using information derived from experimental studies with birds, supported when needed by those conducted with other organisms (mammals, fish and amphibians) and in vitro. The Supporting information details the species, exposure regimes, and main effects of each study conducted on birds in the last 20 years. Extended information on each part of the AOP can be read in the next sub-sections.

Molecular action of triazoles

Triazoles restrain the growth of fungi through the inhibition of the activity of the enzyme lanosterol 14α-demethylase, which synthesizes the ergosterol (an essential component of the fungal wall), disrupting and deteriorating fungal cell membranes and their functionality (Weete 1987). In animals, that enzyme is one of the isoforms of sterol-14α-demethylase (CYP51), which is involved in the biosynthesis of cholesterol, an important lipid that is part of the cell membranes and acts as a precursor of molecules with major physiological functions (Lorbek et al. 2012, Acconcia and Marino 2018). The main MIE of triazoles is the inhibition of the sterol-14α-demethylase, but due to their poor specificity, they also bind to other cytochrome P450 enzymes (Zarn et al. 2003). Different studies have demonstrated that triazoles can inhibit CYP19 aromatases in rats (Zarn et al. 2003, Chen et al. 2019) and frogs (Poulsen et al. 2015), CYP3A4 in humans (Lv et al. 2017), and steroid 17α-monooxygenase (CYP17) in frogs (Poulsen et al. 2015). In vitro assays have shown that CYP17 is also inhibited by different triazoles of pharmaceutical use (clotrimazole, miconazole, ketoconazole and fluconazole) (Munkboel et al. 2019). The molecular processes catalysed by these enzymes are common to all vertebrates. In this context, it has been proven that the docking behaviour of triazoles on aromatases from different species of birds and fish is broadly the same, which suggests that the same molecular mechanisms leading to inhibition of that enzyme could be assumed for all vertebrate organisms (Saxena et al. 2015). Aromatases play key roles in different steps along the steroidogenesis, the metabolic pathway that transforms cholesterol into steroid hormones (i.e. oestrogens, androgens, progestins, glucocorticoids, mineralocorticoids, vitamin D and bile acids) (Acconcia and Marino 2018). Besides, in vitro experiments have evidenced that triazoles can also affect sterol function binding to the membrane protein NPC1, which exports cholesterol from lysosomes to cells (Trinh et al. 2017), and binding to the androgen receptor, which is activated by the union of androgenic hormones (i.e. testosterone or dihydrotestosterone) (Roelofs et al. 2014, Lv et al. 2017).

Cellular responses to triazoles

A direct consequence of triazole-mediated inhibition of the enzyme sterol-14α-demethylase is the reduction of cholesterol synthesis (Acconcia and Marino 2018). Several experimental studies with red-legged partridges exposed to different triazoles used in agriculture (Lopez-Antia et al. 2013, 2018, 2021, Fernández-Vizcaíno et al. 2020) as well as mice exposed to cyproconazole (Peffer et al. 2007) have reported reduced cholesterol levels in plasma. Moreover, triazole binding to the membrane protein NPC1 impedes the transport of cholesterol from lysosomes to cells, reducing the availability of cholesterol as a precursor for other molecules (Trinh et al. 2017, Acconcia and Marino 2018). Thus, reduced cholesterol levels in cells because of disruption of either its synthesis or its transport may alter the steroid hormone homoeostasis, given the role of cholesterol as a precursor of these hormones. In addition, the inhibition by triazoles of other cytochrome P450 enzymes that act on further steps of the hormone biosynthesis process (e.g. aromatase that converts testosterone into oestradiol; Zarn et al. 2003) adds to the effects of these chemicals on the steroid hormone levels in the organisms.

Studies with different model species have reported differences in hormone levels between exposed and non-exposed organisms (either an increase or a decrease). Regarding sexual hormones, Fernández-Vizcaíno et al. (2020) measured lower oestradiol levels in the circulating plasma of both male and female partridges exposed to tebuconazole in comparison to controls. In rats, increased serum testosterone levels were detected after the exposure of adults and peripubertal individuals to different triazoles (Tully et al. 2006, Goetz et al. 2007). In humans, people exposed to triazoles showed reduced testosterone and androstenedione levels in plasma compared to non-exposed ones (Machado et al. 2021). Similarly, exposure of male rats to tebuconazole caused an increase in serum testosterone and a decrease in oestradiol levels (Chen et al. 2019). The direction of triazole effects may be dose-dependent; for instance, in African clawed frogs, low-dose exposure to tebuconazole decreased testosterone, dihydrotestosterone and 17β-oestradiol hormones levels in plasma, while a high-dose also decreased oestradiol but increased testosterone (Poulsen et al. 2015).

As mentioned in the previous section, triazoles may have anti-androgenic effects not only through the biosynthesis pathway alteration but also due to binding to androgen receptors and the subsequent inhibition of testosterone-induced activation of these receptors (Lv et al. 2017). Roelofs et al. (2014) reported a decreased testosterone secretion from murine Leydig cells in vitro due to an inhibition of androgen receptors, and Serra et al. (2023) showed inhibited testosterone secretion from chicken testis cells when they were exposed to different triazoles. In general, this specific mode of action of triazoles altering steroid hormone homeostasis could induce sex-dependent effects not only at the reproductive level but also on other biological functions such as immunity or the antioxidant system (Muriel et al. 2017). This could indirectly influence reproductive success, adding a further layer of complexity to our understanding of the steroid-related effects of triazoles on birds.

Besides all these effects of triazoles on the steroid hormone levels of organisms that are directly exposed, effects on its offspring have also been reported. Fernández-Vizcaíno et al. (2024) found that eggs laid by red-legged partridges exposed to flutriafol had higher estrone levels than those laid by non-exposed partridges, and the same increase was observed for androstenedione levels in eggs laid by partridges exposed to prothioconazole, which may have consequences in the embryo and chick development. That same study reported that partridges exposed to a commercial formulation combining tebuconazole and prothioconazole laid eggs with reduced cholesterol levels (Fernández-Vizcaíno et al. 2024). Whether the potential transgenerational effects are due to a toxic effect because of maternal transfer of the triazoles or just due to a lower transfer of steroids or other key biomolecules (e.g. cholesterol) from the exposed mother to the egg, remains unclear. Despite the low bioaccumulation mentioned above, a certain potential for maternal deposition of triazoles into the egg has been observed, as proven by the detection of epoxiconazole in quail eggs (Grote et al. 2008) and tebuconazole in house sparrow eggs (Bellot et al. 2022a), although some other studies highlight the unfavourable conditions that some triazoles have to be maternally deposited onto avian eggs (Gross et al. 2020, Lopez-Antia et al. 2021). In those scenarios where maternal transfer exists, it should be elucidated whether the transfer may result in triazole doses in eggs susceptible to cause disrupting effects on the sterol synthesis.

The impacts of triazoles on cellular response are not only measurable with endocrine biomarkers but also reflected in a differential gene expression of genes implicated in the biosynthesis and metabolism pathways of sterols and steroids. A pioneering study in birds found that the expression of genes encoding enzymes involved in steroid biosynthesis was overexpressed in comparison to controls when partridges had eaten cereal seeds treated with flutriafol, tebuconazole or prothioconazole (Fernández-Vizcaíno et al. 2020). Additionally, a variety of studies with either adults or embryos of rats have reported results in the same direction. Rat embryos exposed to six different triazoles showed overexpression of the genes related to the steroid biosynthesis pathway, especially at higher doses of difenoconazole and ketoconazole (Dimopoulou et al. 2017a, b). Additionally, Tully et al. (2006) found several differentially expressed genes, mainly genes encoding cytochrome P450 enzymes, transporter genes and genes involved in steroid hormone metabolism, in adult rats exposed to four different triazoles. Similarly, Goetz and Dix (2009) observed that livers of rats exposed to myclobutanil, propiconazole or triadimefon had differential expression of genes involved in fatty acid, steroid and sterol biosynthesis and metabolism pathways as compared to non-exposed ones. Likewise, exposure of rat whole embryo cultures to flusilazole, cyproconazole and triadimefon resulted in differential expression of genes involved in steroid, cholesterol and retinoic acid metabolism pathways (Robinson et al. 2012). In general, as discussed in some of these articles, disruption of steroid homeostasis caused by the triazole mechanism of action, which we have described above, could trigger a compensatory response in the affected organisms that would react by increasing the synthesis of some enzymes or precursor molecules. These compensatory responses could in part explain the differential gene expressions observed in organisms exposed to triazoles.

Tissue and organ responses to triazoles

The binding of triazoles to the androgen receptor inhibits their activation in the presence of testosterone, impeding the progression of spermatogenesis in Sertoli cells (Roelofs et al. 2014, Lv et al. 2017). The alteration of circulating sexual hormone concentrations, as we have reviewed above, can have similar effects. Indeed, Grote et al. (2008) found that the exposure of male quails at two different doses of epoxiconazole resulted in a reduction in the number of spermatids and testicular canaliculi with visible germ cells. Similarly, when testis cells of chicken were exposed in vitro to different triazoles, a decrease in sperm motility and velocity as well as an increased percentage of spermatozoa with abnormal morphology was detected (Serra et al. 2023). In contrast to these findings, Bellot et al. (2023) found that the exposure of male house sparrows to tebuconazole did not cause changes in sperm morphology (head, flagellum and total sperm length). Some studies conducted with other vertebrate groups seem to support the findings from birds pointing to effects on male gonadal structure and function. Thus, the application of triazoles reduced testis size, delayed sperm maturation and accelerated Müllerian duct regression (the process by which Müllerian ducts retract in males, as these ducts are precursors of uterine tissue and only mature in females) of Western clawed frogs after metamorphosis (Svanholm et al. 2021) and decreased sperm motility and altered sperm morphology in rats (Tully et al. 2006). We could not find information about how triazoles affect the reproductive organs of females, which highlights the need for research in this part of the pathway.

Another way of effect at this level relates to the role that cholesterol plays on carotenoid blood transport and deposition in teguments such as skin or beak, so cholesterol levels could influence tissue coloration (McGraw and Parker 2006). Indeed, several studies have shown that triazole-exposed red-legged partridges had a reduced percentage of pigmentation and reduced redness of the eye ring compared to non-exposed ones (Lopez-Antia et al. 2013, 2018, 2021), and some of these exposed partridges had also altered blood cholesterol levels.

Effects of triazoles on reproductive output

The alteration of gonadal development and sperm morphology and function following triazole exposure can ultimately affect the reproductive output of birds. Among other effects, red-legged partridge pairs exposed to a variety of triazoles exhibited a reduction in the fecundation rate of the eggs as well as in the hatching rate (Lopez-Antia et al. 2013, 2021), which altogether caused, in the case of flutriafol-exposed couples, a decreased of more than 50% in the brood sizes compared to non-exposed pairs (Lopez-Antia et al. 2018). Additionally, even though no effects of triazoles on female gonads have been reported yet, red-legged partridges exposed to flutriafol showed a delay in the onset of laying for 14 days relative to controls (Fernández-Vizcaíno et al. 2020) and a reduction in the total number of eggs laid (Lopez-Antia et al. 2018, Fernández-Vizcaíno et al. 2020), while eggs laid by difenoconazole-exposed partridges were smaller than those laid by control couples (Lopez-Antia et al. 2013).

The effects of triazoles on chicks have also been reported. Increased chick mortality during the first days after hatching was observed in broods from red-legged partridges that had been exposed to difenoconazole (Lopez-Antia et al. 2013). Also, direct exposure of eggs to tebuconazole, either by overspray or by incubation in previously sprayed soils reduced chick survival post-hatching (Ortiz-Santaliestra et al. 2020). Sublethal effects on chicks because of parental exposure to triazoles have also been found. For instance, chicks born from grey partridges that had been fed with grain from conventional agriculture (containing pesticide residues of at least four pesticides, including tebuconazole) had a lower body condition than chicks born from partridges that had been fed with grain from organic farming (supposed to be pesticide-free, although no confirmation was conducted) (Gaffard et al. 2022). Lastly, Fernández-Vizcaíno et al. (2024) showed that adult red-legged partridge exposure caused chicks of both the first and second generations to have significant differential expression of genes encoding enzymes involved in sterol biosynthesis compared to non-exposed individuals.

An additional aspect of effects on avian reproduction that has not been investigated, because the studies referred above use artificial incubators and hatchers for the eggs, is the effect that a potential alteration of incubation behaviour may have on breeding success. Behavioural effects of triazoles on birds have hardly been investigated, but Jackovitz et al. (2018) revealed effects of nitrotriazolone (a triazole that has not been used as agricultural fungicide) on neuromuscular function that came along with histopathological alterations in the brain of Japanese quails. However, whether these neurological effects could affect incubation behavior (and hence add a level of effect that would pass unnoticed in experimental assays using artificial incubators) remains unknown.

The effects of triazoles on ornamentation can also scale up to the reproductive process. Alonso-Alvarez et al. (2012) experimentally observed that female red-legged partridges increased their clutch size when mating with males with more intense redness of beak and eye rings, which means that females can adjust their breeding investment according to male carotenoid-based ornamentation because this honestly reflects its individual quality and health status (Blas et al. 2006, Pérez-Rodríguez et al. 2013).

Other triazole impacts outside the reproductive AOP

Experimental studies with triazoles and birds have revealed some detrimental effects that, even if not directly linked to the AOP processes described, may also affect their breeding process. At the cellular level, triazoles can provoke thyroid endocrine disruption. In sparrows exposed to tebuconazole, thyroxine (T4) levels decreased in comparison to non-treated birds (Bellot et al. 2023). Even though triiodothyronine (T3) concentrations did not change in response to the treatment, the hypothalamic-pituitary-thyroid axis seemed to be disrupted after triazole exposure, but the specific mechanism for this effect is still unknown (Bellot et al. 2023). Red-legged partridges exposed to a commercial mixture of tebuconazole and prothioconazole showed elevated plasma activities of the enzymes aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) (Fernández-Vizcaíno et al. 2020), which would be indicative of liver damage. Although no specific mechanism has been proposed for how triazole exposure could cause liver damage in birds, research in fish has shown that effects on this organ could be related to changes in lipid and lipoprotein homeostasis, oxidation of biomolecules and altered metabolism of fatty acids and ketone bodies (Huang et al. 2022). Thus, the metabolism of triazoles by hepatic enzymes could be affecting liver tissues. In this context, the oxidative status of red-legged partridges seems to be altered by triazole exposure as revealed for instance by increased superoxidase dismutase (SOD) activity in partridges exposed to difenoconazole through treated seed consumption (Lopez-Antia et al. 2013). This effect on oxidative status is likely derived from oxidative detoxification side-effect costs (Ronis and Badger 1995), hence the liver, as the main organ for triazole metabolism, could be suffering from the damage caused by free radicals resulting from detoxification processes.

Other plasma biochemistry parameters have also been shown to be affected by triazole exposure in birds. Uric acid and phosphorus concentrations (Fernández-Vizcaíno et al. 2020) were higher in partridges exposed to prothioconazole (and to flutriafol for the uric acid case) than in controls, which could also indicate renal damage. Also, plasma levels of calcium, magnesium, albumin, total proteins, triglycerides (Lopez-Antia et al. 2018, 2021) and retinyl palmitate (vitamin A) (Lopez-Antia et al. 2013) were lower in triazole-exposed partridges than in control ones. These effects have also been observed in eggs from exposed partridges, which had higher yolk retinol levels when exposed to prothioconazole and lower tocopherol yolk level when exposed to a mixture of tebuconazole and prothioconazole, than eggs from non-exposed partridges (Fernández-Vizcaíno et al. 2024).

Effects of triazoles that are not necessarily related to the AOP are also exhibited at the tissue and organ levels. For example, Fernández-Vizcaíno et al. (2020) found that female red-legged partridges exposed to prothioconazole had higher haematocrit than controls, possibly due to dehydration from the abovementioned renal damage. Moreover, Bellot et al. (2023) reported that exposed females of house sparrows regrew larger but less dense feathers than controls. At the organism level, Bellot et al. (2022b) found that the resting metabolic rate of exposed sparrows was significantly lower than in controls at thermoneutrality at 25°C but failed to find differences in the thermoregulatory metabolic rate at 12°C between exposed and control individuals. Moreover, Grote et al. (2008) reported that quail males exposed to epoxiconazole had a lower body weight gain than controls, which contrasts with the fact that most of the studies have not detected differences in body condition between exposed and non-exposed birds (Lopez-Antia et al. 2018, 2021, Fernández-Vizcaíno et al. 2020, Gaffard et al. 2022, Bellot et al. 2023, 2024). Overall, all these responses at different levels of biological organization could reflect a compromise between the maintenance of general physiological functions while metabolizing and detoxifying the triazoles that are absorbed by organisms. Thus, even if these effects are not directly related to reduced reproductive outcomes, limited resources available to exposed organisms might be balancing the situation towards a costlier reproduction.

Conclusion

The results reported from experimental studies highlight the impacts of triazoles on avian reproduction and fitness. The AOP proposal presented in this review is still far from being complete, as there are many key event relationships that are yet to be elucidated to fully understand the mechanisms through which triazoles affect bird reproduction. In addition to the inherent complexity of the mechanisms being studied, research has been carried out in both wild and not-wild species, involving different triazoles types, with a variety of exposure times and concentrations. This diversity of parameters makes it difficult to compare studies, but the present review serves to help us contextualizing the results obtained to date, to identify the knowledge gaps and to highlight that these negative outcomes and long-term effects on birds should motivate a closer look at possible gaps in pesticide regulation. As shown in Fig. 1, the information contained in the combination of available studies generates an AOP proposal with a high degree of completeness. Further research needs in this context go in two main directions; on the one hand, filling the gaps in key events (the boxes in Fig. 1) of the AOP, in particular what refers to the effects on female gonads. On the other hand, characterize the key events relationships (the arrows in Fig. 1); although these KER are supported by experimental results, more empirical data to quantify the ranges at which key events relationships happen and their dose-dependence are needed. Overall, the triazole case triggers the need to consider whether current risk assessment protocols are enough to comprehend the complexity of the effects observed and if these protocols are efficient to protect birds living in agricultural habitats from anthropic impacts.

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Supporting information

The Supporting information associated with this article is available with the online version.

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