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Anticoagulant rodenticides and resistance development in rodent pest species – A comprehensive review

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ABSTRACT

Anti-vitamin K (AVK) compounds are highly potent anticoagulants which are particularly effective for controlling rodent species populations. AVKs have been the most widely used chemical rodenticide option employed since the 1950s for the control of rodents infesting stored commodities and storage facilities, and also in a wide range of other scenarios. However, reports of AVK resistance in wild rodent populations are becoming increasingly common. This could potentially lead to a substantial reduction in AVK efficacy resulting in an impaired ability to manage rodent infestations in the future. The current state of knowledge regarding AVK resistance mechanisms in common pest species is still incomplete. This review draws together reported incidences of AVK resistance in the literature and the underlying mechanisms suspected of conferring resistance for the three main pest rodent species *Rattus norvegicus*, *Rattus rattus* and *Mus musculus*. The purpose of this review is to compare and contrast the underlying resistance mechanisms in these species and demonstrate how this should influence programs for monitoring and avoiding the development of AVK resistance in target rodent species.

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Contents

1. Introduction 2
2. Anti-vitamin K (AVKs) 2
   2.1. Development of AVK compounds 2
   2.2. First generation anti-coagulants rodenticides (FGARs) 2
   2.3. Second generation anti-coagulant rodenticides (SGARs) 3
   2.4. AVK mechanism and mode of action 3
   2.5. AVK baits 4
3. Rodenticide resistance 5
   3.1. Rodenticide resistance mechanisms 5
   3.2. Behavioural avoidance 5
   3.3. Vkorc1 gene 5
   3.4. Introgressed hybridisation 5
   3.5. Natural resistance 5
   3.6. Gender 6
   3.7. Pharmacokinetics: P-450 cytochrome sub-families 6
   3.8. Calumenin 6
   3.9. Microbiome 6
4. Biological cost of AVK resistance 6
5. Techniques for determining resistance 7
   5.1. Lethal feeding period test 7
   5.2. Blood clotting response (BCR) 7

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5.3. Kinetic studies on VKOR activity 7
5.4. Molecular characterisation of resistance − vkorc1 gene 8
6. Known resistance mechanisms in rodent species 9
6.1. Rattus norvegicus 9
6.2. Rattus rattus 11
6.3. Mus musculus 13
7. Conclusion 14
Declaration of competing interest 15
Acknowledgements 15
Supplementary data 15
References 15

1. Introduction

Measures for the safe and effective control of rodent populations are of critical global importance in developed and developing countries alike (Jacob and Buckle, 2018). In rural areas rodent species are responsible for substantial damage to cereal and other crops, whilst urban rodent infestations pose considerable threats to public health and hygiene as they can be vectors of numerous pathogens including plague, haemorrhagic fever, Weil's disease and the hepatitis E virus to name but a few (Goulois et al., 2017a; Ishizuka et al., 2007; Meerberg et al., 2009; Morand et al., 2009; Singla et al., 2008). Rodents are, of course, significant pests of almost all stored commodities to which they are able to obtain access (Brooks and Fiedler, 1999; Stejskal et al., 2015).

Control of rodent populations, in storage facilities and elsewhere, can take many forms including both physical traps and chemical control measures. Chemical control is most often performed using active substances which disrupt vitamin K cycling, these compounds are conventionally now referred to as anti-vitamin Ks and commonly abbreviated to AVKs (Feinstein et al., 2016; Vein et al., 2013). Chemical control using AVKs was first established in the late 1940s through the development of warfarin and dicoumarin derivatives which are now generally referred to as first generation anticoagulant rodenticides (FGARs) (Wardrop and Keeling, 2008). FGARs were found to be capable of inducing haemorrhaging in rodents by blocking the vitamin K cycling pathway leading to excessive bleeding and eventual death (Buckle and Eason, 2015). However, as early as the 1950s, resistance to these rodenticide compounds was documented in wild rodent populations (Pelz and Prescott, 2015; Berny et al., 2018). The development of resistance has been an on-going problem with AVK rodenticides throughout their usage. In the 1970 and 1980s a so called "second generation" of anticoagulant rodenticides (SGARs) was developed. SGARs are structurally similar to FGARs but contained converted side chains which resulted in greater efficacy against rodents and counteracted some of the FGAR resistance which had developed in certain commensal rodent pest populations (Feinstein et al., 2016). However, resistance to SGARs has increasingly been documented in wild populations across wide ranging geographical locations (Mooney et al., 2018; Quy et al., 1995). Given these pressures, the aim of this review is to outline the current understanding of FGAR and SGAR resistance development in wild rodent populations and what it means for the future of pest control, both in commodity storage facilities and more widely elsewhere. This review will have a particular focus on developments since 2004 when the *vkorc1* gene was identified and understood to be highly influential in mediating AVK resistance (Pelz et al., 2005). The scope of this review will encompass the three most successful globally distributed rodent pest species *Rattus norvegicus, Rattus rattus* and *Mus musculus* (Puckett et al., 2016).

2. Anti-vitamin K (AVKs)

2.1. Development of AVK compounds

The isolation and characterisation of the anticoagulant compound dicoumarin in the 1940s marked the beginning of the era of the AVK rodenticides (Feinstein et al., 2016). Dicoumarin was identified as the causative agent of "sweet clover disease" a haemorrhagic disease of cattle widely observed in the USA and Canada in the 1920s and studied in the laboratory of Karl Link (Last, 2002; Stahman et al., 1941). Interest in the anticoagulant properties of dicoumarin and its derivative compounds was primarily driven by an interest in discovering a treatment for the medical condition thrombosis (Wardrop and Keeling, 2008). One of the most potent dicoumarin derivatives developed was warfarin, a compound whose anticoagulant property is still widely used for treating thrombosis today (Porath et al., 2019). However, it was not long before the potential for using AVK compounds as rodenticides was recognised. Trials assessing the suitability of dicoumarin derivatives and warfarin as rodenticides were conducted throughout the 1940s and demonstrated impressive efficacy (Wardrop and Keeling, 2008). This led to the concept of using warfarin as a rodenticide being widely promoted in the mid-1940s (O'Connor, 1948). Subsequently, this led to the registering of the compounds dicoumarin and warfarin as rodenticides in the UK and USA in 1949 and 1950, respectively (Hadler and Buckle, 1992). Dicoumarin and warfarin have since come to be regarded as the first FGARs and within a few years several other similar compounds were added to this class of rodenticides.

2.2. First generation anti-coagulants rodenticides (FGARs)

Currently, all AVK rodenticides are derivatives of two compounds, 4-hyroxycoumarin and indane-1,3-dione, the chemical structures of both are shown alongside coumarin and warfarin in Fig. 1 (Pelz et al., 2005). Warfarin and dicoumarin are 4-hydroxycoumarin-based FGARs and since their discovery several additional 4-hydroxycoumarins FGARs have been developed including coumachlor (1951), coumafuryl (1953) and coumatetralyl (1956) (Hadler and Buckle, 1992). The FGAR class of rodenticides also includes several indane-1,3-dione-based rodenticides such as pindone (1940s) diphacinone (1952) and chlorophacinone (1961) (Hadler and Buckle, 1992; Jolly et al., 1994). Despite being on the market since the 1940s, many FGARs, such as warfarin are still routinely used in treating rodent infestations, however, resistance to FGARs has become a significant problem in many target species (Suarez and Cueto, 2018).

FGARs are considered to be chronic toxicants, as repeated consumption of small quantities over a protracted time period results in rodent mortality (King and Tran, 2015). Repeated consumption of

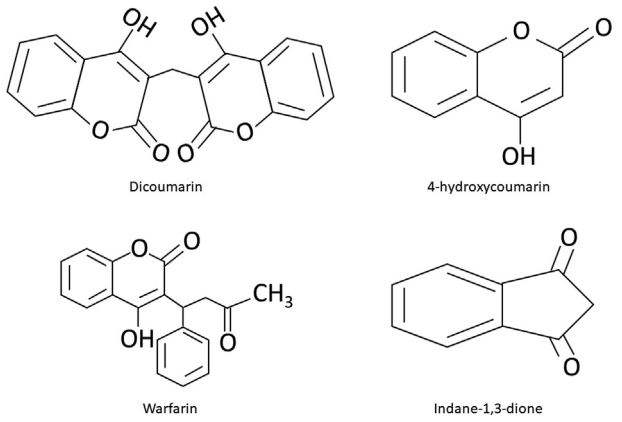

Fig. 1. Chemical structures of dicoumarin, warfarin and the AVK "parent" compounds 4-hydroycoumarin and Indane-1,3-ione.

FGARs is necessary to sustain an effective concentration in rodents to induce mortality (Matagrin et al., 2013). This is because FGARs have relatively short biological half-lives due to the efficiency of the rodent cytochrome P-450 detoxification system (Boitet et al., 2018; Markussen et al., 2008). As a result of the relatively rapid clearance rate for FGARs the concentration of warfarin required for an acute poisoning (rat acute LD_{50}: 10–323 mg/kg) is much greater than the concentration required to induce mortality through sub-acute poisoning (sub-acute LD_{50} in rats: 5 doses of 1 mg/kg) (Hadler and Buckle, 1992; Buckle and Eason, 2015). However, the high palatability of modern rodenticide baits, plus lack of short-term adverse effect, serves not to deter rodent species feeding on baits containing AVK poisons repeatedly (Suarez and Cueto, 2018).

Nowadays FGARs are generally considered to be less efficacious than SGARs because they require multiple feeding events to induce mortality in target rodents. Additionally, resistance is more prevalent for FGARs compared to SGARs (Rattner et al., 2014; Suarez and Cueto, 2018). However, in non-resistant rodent populations both FGARs and SGARs can be equally suitable for eradicating rodent infestations.

2.3. Second generation anti-coagulant rodenticides (SGARs)

The majority of current SGARs are modified versions of the 4-hydroxycoumarin compound (Watt et al., 2005), though one SGAR, difethialone, is a derivative of benzothiopyranone, a compound similar to 4-hyroxycoumarin (Berny, 2011). SGARs were first created in the 1970s by substituting phenyl ring side-chains onto the 4-hydroxycoumarin moiety (Buckle and Eason, 2015; Feinstein et al., 2016; Hadler and Buckle, 1992). This subsequently led to the creation of the so called "superwarfarin" coumarin-based SGAR compounds; brodifacoum, bromadiolone, difenacoum, flocoumafen and the benzothiopyranone-based difethialone (Berny, 2011; Rattner et al. 2014). These SGARs were found to be far more efficacious for controlling rodent species than the original FGARs and could overcome the resistance that some wild rodent populations had built up against certain FGAR compounds (Berny et al., 2018).

Initially, it was first hoped that SGARs would induce acute poisoning in target rodents after one feeding event (single application), but early field trials indicated that complete control of rat populations was not achieved (Hadler and Buckle, 1992). This led to a new baiting technique for controlling rodent infestation called "pulsed baiting" where SGAR baits are replenished at 7-day intervals until control of rodent infestations is achieved. This technique proved to be more effective than previous approaches (Buckle and Eason, 2015; Dubock, 1982).

SGARs had many advantages over FGARs, firstly they can be used at much lower concentrations than FGARs reducing the chances of rodents associating illness to rodenticide baits, thus limiting any bait shyness characteristics developing in target rodent populations (Saurez and Cueto, 2018). Secondly, the modified side chains of SGARs made these compounds more lipid soluble, thus increasing their retention in rodent tissues resulting in longer biological half-lives leading to greater potency (Mooney et al., 2018). However, this characteristic also results in potential risks to non-target wildlife which SGARs are known to pose (van den Brink et al., 2018). Though the chemical structures of SGARs and FGARs differ, the physiological effects the two classes of AVK rodenticides have on rodents are similar.

2.4. AVK mechanism and mode of action

The main site of action for AVKs is in the liver where vitamin K is utilised in the production of blood clotting factors which are essential for coagulation (Feinstein et al., 2016). Both FGAR and SGAR compounds possess a similar mechanism of action (MOA) whereby target rodents are affected on a molecular level (i.e. the biochemical response) resulting in an identical mode of action (MoA), which is the anatomical change resulting from exposure (i.e. the physiological response) (Littin et al., 2000). The AVK rodenticide MOA and MoA has been thoroughly elucidated since their discovery and has subsequently been quite sufficiently reviewed (Berny, 2011; Buckle and Eason, 2015; Hadler and Buckle, 1992; Horak et al., 2018), only a brief overview of the process will be given herein.

FGAR and SGAR compounds act by restricting the bioavailability of vitamin K thus limiting its utilisation in biochemical pathways (Hadler and Buckle, 1992). Vitamin K dietary intake is often insufficient for physiological needs, hence the body recycles vitamin K epoxide to provide an adequate supply (Lefebvre et al., 2016). FGAR and SGAR toxicity can actually be treated by providing an elevated intake of vitamin K (whether by diet or intravenous administration) which is sufficient to reverse the physiological effects of AVK rodenticide poisoning (Spahr et al., 2007). In certain rodent species, such as the beaver *Aplodontia* rufa, restricting the access to sources of vitamin K has been shown to increase the efficacy of AVK rodenticides (Arjo and Nolte, 2004) However, not all rodent species appear to benefit from a source of vitamin K while exposed to AVK rodenticides. Providing an additional source of dietary vitamin K to the Indian gerbil (*Tatera indica*) prolonged the time until death, but mortality still occurred (Chaudhary et al., 2004). The Montane vole (*Micotus montanus*) was also shown to be controllable using the FGARs containing baits (chlorophacinone and diphacinone) even with access to a vitamin K_1 rich diet (Witmer et al., 2013). In *R. norvegicus* access to dietary vitamin K has been shown to prevent an increase in blood clotting times in vitamin K deficient *R. norvegicus* (Jacob and Freise, 2011). However, access to diet rich in vitamin K has been shown to have no effect on the efficacy of brodifacoum and diphacinone pellets used for controlling populations of *R. norvegicus*, *R. rattus* and *M. musculus* (Witmer and Burke, 2009). One explanation for this could be provided by the study of MacNicoll and Gill (1993) which demonstrated that AVK-resistant *R. norvegicus* had an increased survival rate when provided with a diet rich in vitamin K_3 (mendione sodium bisulphite) when exposed to AVKs (bromadiolone, brodifacoum, flocoumafen and difenacoum). The vitamin K_3 essentially acted as an antidote to the AVK poisoning in the AVK-resistant rats. Interestingly, the same effect was not observed in the non-AVK resistant rat population nor in resistant or non-resistant *M. musculus* populations. This indicated that a source of

dietary vitamin K may be beneficial but only in AVK-resistant rats.

The specific AVK MOA was first elucidated in 1973 when Whitlon et al. (1978) showned that coumarin compounds inhibit the vitamin K epoxide reductase enzyme (VKOR). The function of VKOR is to convert vitamin K epoxide into a bioactive form (vitamin K hydroquinone) which is incorporated into several different vitamin K-dependent blood clotting factors (II, VII, IX and X) involved in the blood clotting process as shown in Fig. 2 (Lefebvre et al., 2016; Rost et al., 2009). When AVKs block the VKOR enzyme, the resulting lack of vitamin K-dependent blood clotting factors results in the AVK MoA whereby a lack of prothrombin (complex containing blood clotting factors II, VII, IX and X) results in rodents perishing through excessive internal bleeding i.e. haemorrhaging, or also through external bleeding from orifices or skin lesions if present (Suarez and Cueto, 2018; Watt et al., 2005).

2.5. AVK baits

AVK compounds can be incorporated into a wide range of differing types of bait product for attracting rodent species. Rodenticide baits come in many different physical forms each of which generally have distinctly different chemical formulations. Commonly-used AVK rodenticide bait types employed in the control of mice and rats include wax blocks, pastes, pellets and grains, though other forms also exist (Suarez and Cueto, 2018). Rodenticide bait formulations generally contain several ingredients in addition to the active substance; these include solvents, bittering agents (such as denatonium benzoate), diluents, stabilizers, additives, co-adjuvants, preservatives and inert base substances which preserve the chemical and physical properties of the bait formulation (Berny et al., 2014; Suarez and Cueto, 2018). Modern AVK rodenticide formulations are expected to be able to withstand weathering and adverse environmental conditions while in place and still remain highly palatable (Nakagawa et al., 2015). Bait products in the EU have typically contained 50 ppm of the SGAR AVK active substance, however, recently products with concentrations <30 ppm have been authorised for placement on the market. Though there is little information publicly available regarding the efficacy of these lower concentration baits, the study of Frankova et al. (2019) demonstrated that 25 ppm brodifacoum baits demonstrated the same level of efficacy as 50 ppm baits against non-resistant *M. musculus*. There are also reports of rodenticide baits containing as little as 10 ppm of bromadiolone being effective against *R. rattus* (Garg and Singla, 2016).

The main component of a bait formulation is generally the base; a carbohydrate source which attracts the target rodents. The formulation of baits is highly important for enhancing acceptance by target species and also in ensuring that they remain highly palatable to target organisms. Studies have shown that certain bait types perform better than others at attracting target species and this may be due to the formulation type and commercial supplier (Suarez and Cueto, 2018). The type of bait may also play a role in acceptance by target rodent species (Singla and Parshad, 2002). The choice of the base carbohydrate should ideally be tailored to suit the environment in which they are expected to be used, such as the target rodent population's traditional food source (Leung et al., 2007). Acceptance of baits by wild rodent species has been shown to be highly influenced by the presence or absence of alternative food sources and the location of their placement on sites with infestations (Quy et al., 1996). Acceptance of AVK baits in certain locations can be extremely low when there is an abundance of alternative food sources present and the resulting failure to reduce rodent numbers after application of the rodenticides could be mistaken for behavioural resistance (Quy et al., 1992).

When placing rodenticides in open areas for long durations, it is generally recommended to use bait stations. Bait stations are structures used to contain bait which is either physically tethered or loosely applied inside and allow rodents access. This limits the amount of bait being dragged out into the environment and allows for monitoring of consumption. Certain characteristics of bait stations have been shown to influence the level of acceptance of the bait contained inside. The study of Volfova et al. (2010) demonstrated that mice preferred bait stations containing either male or female scents as opposed to cleaned bait stations. Different tamper resistant bait station designs have been shown to deter bait consumption in *R. norvegicus* populations compared to bait left in open trays but otherwise protected from non-target animals using materials found at the treated site (Buckle and Prescott, 2010). However, bait stations are essential in reducing bait release into the environment and exposure to the general public.

The application of AVK bait and acceptance by target rodent species has also been shown to alter rodent behaviour in the days

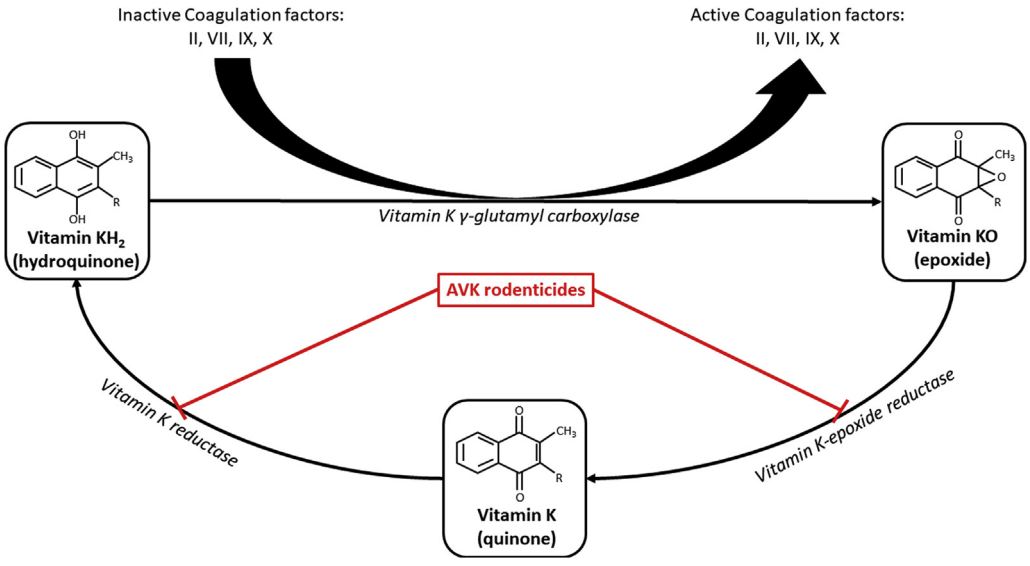

Fig. 2. Representation of biochemical vitamin K metabolism, the activation of coagulation factors and target sites of AVK rodenticides.

after consumption. Studies performed on both *R. norvegicus* and *M. musculus* have demonstrated that after consumption of AVK baits, rodent cover seeking behaviour (thigmotaxis) is altered and their light-dark rhythm can become unbalanced. In the laboratory study conducted by Cox and Smith (1992) brown rats which consumed AVK pellets were found to spend more time away from cover and out in open spaces, particularly so during "daylight" hours. The authors hypothesised that this behaviour may have resulted from blindness developing from AVK poisoning and the likely result in the wild would increase chances of predation occurring on poisoned rats. The study of Brown and Singleton (1998) supported these findings in a trial conducted on wild *M. domesticus* populations infesting Australian wheat crops. In their study *M. domesticus* which consumed bait pellets containing brodifacoum were found to alter their activity patterns increasing the distance they ranged and becoming nomadic and not site-attached. Consumption of AVK baits has also been shown to affect rodent feeding behaviour, typically after consuming AVK baits, rats and mice tend to have reduced food consumption. The studies of both Littin et al. (2000) and Frankova et al. (2017) found rodent appetite to be suppressed prior to the occurrence of mortality. These studies indicate that using AVK baits to control target rodent pests alters behaviour and increases the chances of secondary poisoning in organisms, such as birds of prey, which consume rats and mice.

3. Rodenticide resistance

3.1. Rodenticide resistance mechanisms

AVK resistance has been found to have evolved in numerous mouse and rat populations across the world in response to their use (Rost et al., 2009; Pelz and Prescott, 2015; Berny et al., 2018). When considering what constitutes AVK resistance in wild rodent populations, perhaps the best definition was given by Greaves (1994) where he defined it as "a major loss of efficacy in practical conditions where the anticoagulant has been applied correctly, the loss of efficacy being due to the presence of a strain of rodent with a heritable and commensurately reduced sensitivity to the anticoagulant". However, various rodent species have been shown to possess a variety of AVK resistance mechanisms ranging from behavioural avoidance of baits to the evolution of molecular resistance to AVK compounds (Berny et al., 2018). This review will provide a broad overview of the many types of resistance that have been identified to date but will particularly focus on the growing understanding of the genetically-mediated development of AVK resistance in each of the three common rodent pest species *R. norvegicus*, *R. rattus*, and *M. musculus*.

3.2. Behavioural avoidance

Certain rodent populations have been shown to evade AVK poisoning by simply avoiding consumption of rodenticide products (Brunton et al., 1993). Behavioural avoidance of rodenticide baits stems from a neophobic trait (a fear of unfamiliar items in an environment) often referred to as "bait shyness" or "food aversion" that is considerably more pronounced in rat species than in mice (Barnett, 1958; Berny, 2011; Hadler and Buckle, 1992). Rodent species have been shown to avoid new objects, such as rodenticide bait boxes in their environment even when there is a highly palatable AVK rodenticide present inside (Quy et al., 1992: Buckle and Prescott, 2010). Behaviourally, rodents have been shown to generally consume only a portion of the bait initially before returning at a later point once they have not associated any adverse effects to the new food source (Suarez and Cueto, 2018). AVK rodenticides partially owe their success to their lack of acute toxicity which prevents rodents associating toxicity with AVK baits, thus overcoming their neophobic traits (Berny, 2011). Producing highly palatable rodenticide baits is key when trying to overcome bait aversion.

3.3. Vkorc1 gene

An inheritable molecular basis of AVK resistance in rodents had long been known. The warfarin resistance gene in *R. norvegicus* had been narrowed down to chromosome 1 as early as 1967 (Greaves and Ayres, 1967) and the warfarin resistance gene in the house mouse was linked to chromosome 7 in 1976 (Wallace and MacSwiney, 1976). The VKOR multiprotein complex was known to be the biological target inhibited by AVKs in 1973 (Whitlon et al., 1978), however, it wasn't until 2004 that the specific gene which encodes the VKOR protein was identified in the human genome by Rost et al. (2004). The site of warfarin resistance was found to be specifically located on the vitamin K epoxide reductase complex subunit 1 (*vkorc1*) gene, which encoded a subunit of a transmembrane protein in the endoplasmic reticulum (Rost et al., 2004). The work of Pelz et al. (2005) demonstrated that rodents possessed a homologous *vkorc1* gene and that it also influenced AVK resistance. Since the identification of this gene, much research has focused on identifying *de novo* single nucleotide polymorphism (SNPs) which alter the amino acid sequence of the protein conferring resistance to FGAR and SGAR compounds (Rost et al., 2009). To date, several *vkorc1* SNPs which enhance AVK resistance have been shown to have evolved independently in different rodent populations across the world demonstrating how large rodent populations have responded similarly to the selection pressure introduced through usage of AVKs (Goulois et al., 2017a).

3.4. Introgressed hybridisation

Several studies have identified an instance where a cross hybridisation event between two mice species has led to increased levels of AVK resistance (Goulois et al., 2017a; Song et al., 2011). This mechanism of resistance was first postulated by Kohn and Endepols (2009). Subsequently, certain AVK-resistant populations of the mouse species *M.m. domesticus* have been shown to have originated from interbreeding with the Algerian mouse *Mus spretus* (Song et al., 2011). Cross breeding between the mice species led to an introgression of a >10 Mb section of the *M. spretus* chromosome 7 containing an allele for the *vkorc1* gene ($vkorc1^{spr}$) into the *M.m. dometicus* genome (Song et al., 2011). The $vkorc1^{spr}$ has been associated with conferring strong resistance in the resulting offspring of *M. spretus* - *M.m. domesticus* hybrid populations (Song et al., 2011). A study by Pelz et al. (2012) found the group of *vkorc1* mutations (Arg12Trp/Ala26Ser/Ala48Thr/Arg61Leu) linked to *M. spretus* to be widespread across *M. musculus* populations in Germany. Though the Pelz et al. (2012) study found the Arg61Leu substitution mutation to be less tightly linked to the *M. spretus* genotype than the other 3 mutations and was sometimes absent in *M. musculus* which contained the Arg12Trp, Ala26Ser and Ala48Thr mutations. Goulois et al. (2017a) later established in *in vitro* yeast recombinant studies the level of AVK resistance conferred in the resulting VKOR protein produced by these 4 introgressed SNPs which contribute to the strongly resistant phenotype of the $vkorc1^{spr}$ in *M.m. domesticus*.

3.5. Natural resistance

Rodent species differ widely in their intrinsic susceptibility to anticoagulants and Greaves (1994) coined the term 'natural resistance' to describe this phenomenon. For example, the Egyptian

spiny mouse (*Acomys cahirus*), Shaw's jird (*Meriones shawi*) and the Syrian hamster (*Mesocricetus auratus*) have been found to be relatively insensitive to a wide range of anticoagulant rodenticides, and the former is almost impervious to many of them (Greaves, 1994). All are xerophilous species and it may be something about life in desert habitats that mitigates towards this physiological trait. Even among the more traditional rodent pest species, *R. norvegicus*, *R. rattus* and *M. musculus*, there are significant differences in levels of intrinsic susceptibility to anticoagulants (Buckle and Eason, 2015), with the house mouse possessing a high degree of natural resistance. Physiological mechanisms that underlie natural resistance are poorly understood and presently can only be speculated upon. However, likely candidates conferring natural resistance appear to be differences in the physiology of the vitamin K cycle, the blood clotting mechanism and differences in the efficiency of mechanisms for the elimination of toxins, such as the P-450 cytochrome systems described in the next section.

Natural resistance should not be confused with true resistance phenomena but problems of treatment efficacy are likely to be exacerbated when natural resistance is superimposed upon one of the more well-known resistance mutations. This may explain why, among the common rodent pest species, anticoagulant resistance in house mice is very widely encountered.

3.6. Gender

It has long been known that gender plays a role in AVK resistance in many rodent species. The study of Buckle et al. (2007) reported greater resistance to bromadiolone in female Norway rats than their male counterparts in a resistant wild population found on a Welsh farm. Female rats have also been shown to be more resistant to difenacoum(Greaves and Ayres, 1988). Kerins and MacNicoll (1999) found after administering brodifacoum to both male and female rats, that longer biological half-lives of certain blood clotting factors in females may play a role in the gender-based AVK tolerance mechanism. A study conducted by Lefebvre et al. (2016) confirmed that a stronger pool of available vitamin K-dependent blood clotting factors resulting from a slower decline in the levels of blood clotting factors was characteristic of female rats administered with difethialone. The Lefebvre et al. (2016) study also found that pharmacokinetic and VKOR activity were not the source of difethialone resistance in female rats. However, differences in pharmacokinetics have been highlighted between male and female rats in certain cases, higher expression of certain P-450 cytochromes was observed in female bromadiolone-resistant rats than in males (Markussen et al., 2008). Garg and Singla (2014) also found that females of the species *Rattus rattus* were more resistant to difenacoum and bromadiolone than males.

The influence of gender on AVK resistance in house mice has been known since the 1970s when the resistance gene was pinpointed to chromosome 7 on the house mouse genome (Wallace and MacSwiney, 1976). Bromadiolone resistance in a wild mouse population in Serbia was also shown to be influenced by gender in a study by Scepovic et al. (2016). This study also identified a genetic link to resistance in the *M. musculus* population and clearly identified a greater degree of tolerance to bromadiolone in female mice than their male counterparts.

3.7. Pharmacokinetics: P-450 cytochrome sub-families

Several studies have demonstrated how pharmacokinetic characteristics can mediate a level of AVK resistance in rodent species (Boitet et al., 2018; Ishizuka et al., 2007; Markussen et al., 2008; Takeda et al., 2016). Several sub-families of the P-450 cytochromes are known to mediate AVK metabolism (Daly and King, 2003). A study by Ishizuka et al. (2007) demonstrated warfarin resistance in roof rats in Japan (*R. rattus*) which did not possess any known *vkorc1* AVK resistance SNPs. Their study found that warfarin-resistant rats had higher P-450 cytochrome activity and resistance was associated with the resulting increased clearance capability within this species. Takeda et al. (2016) further elucidated the cytochrome resistance mechanism in *R. rattus* demonstrating specifically which P-450 sub-families were involved in the hydroxylation of warfarin, facilitating its clearance.

Over-expression of certain P-450 cytochrome sub-families has also been indicated as a source of AVK tolerance in *R. norvegicus* (Boitet et al., 2018; Markussen et al., 2008). Markussen et al. (2008) demonstrated that cytochrome activity contributed to bromadiolone resistance in Norway rats carrying the Tyr139Cys mutation in the *vkorc1* gene. Additionally, the Boitet et al. (2018) study showed that elevated cytochrome activity, in conjunction with the Leu120Gln mutation in the rat *vkorc1* gene, contributed to difenacoum resistance.

3.8. Calumenin

Wajih et al., 2004 revealed how the calcium-binding protein calumenin could also play a role in AVK resistance. Calumenin is an endoplasmic reticulum-bound chaperone protein involved in the γ-carboxylation system of vitamin K-dependent proteins. In the Wajih et al., 2004 study silencing the calumenin gene was found to reduce carboxylation activity, thus inhibiting vitamin K cycling. The Wajih et al., 2004 study also identified a warfarin-resistant laboratory strain of *R. norvegicus* which over-expressed calumenin despite having a *vkorc1* sequence identical to a warfarin-sensitive strain. They hypothesised that alternative genetic AVK resistance mechanisms to *vkorc1* SNPs and P-450 cytochrome activity exist in rodents.

3.9. Microbiome

The potential for pesticide resistance originating from the microbiomes present in the gut microbiome (i.e. the microbial consortia present in the gastrointestinal tract) of target organisms such as rodents is becoming increasingly apparent (Gressel, 2018). The gut microbiome can be considered to be the total genetic potential of the microbes present in the digestive system i.e. the functions which they are capable of performing such as vitamin K cycling (Amon and Sanderson, 2017). The mammalian microbiome is known to produce vitamin K which is subsequently absorbed by the host, providing a steady supply of vitamin K which can mitigate the toxic effect of AVKs (Gressel, 2018). A study by Chuang et al., (2014) demonstrated that transplanting microbiome inoculations from humans treated with warfarin to germ free Webster mice conferred a degree of warfarin resistance in these mice, possibly by introducing microbial strains primed for increased vitamin K production. Additionally, human patients treated with antibacterial compounds whilst on a stable warfarin regime have been known to suffer warfarin over-doses possibly resulting from the impaired microbial activity in their gut resulting in a reduced supply of vitamin K to the host (Glasheen et al., 2005). It is therefore possible that the microbiome plays a role in conferring AVK resistance. However, this area is still in its infancy and more research is necessary before concrete conclusions can be drawn.

4. Biological cost of AVK resistance

One commonly-found consequence of genetically acquired resistance in various species is that the conferred advantage against a toxic substance often comes alongside an undesirable biological

cost (Vogwill and MacLean, 2015; Pelz and Precott, 2015). This is not necessarily always the case but there have been many observed examples across a range of organisms. In rodent species, the cost of acquired AVK resistance has remained relatively under-investigated, though, there is evidence that certain unfavourable biological costs exist. A degree of selective pressure against maintaining AVK resistant phenotypes was highlighted in wild *R. norvegicus* populations in the UK as early as the 1970s (Greaves et al., 1977). Another study has reported that homozygous AVK-resistant strains of *R. norvegicus* have reduced growth rates and possess smaller body sizes compared to heterozygous resistant strains (Smith et al., 1991). Resistant *R. norvegicus* populations have also been shown to have higher vitamin K dietary requirements as a result of the impaired vitamin K metabolism associated with certain *vkorc1* SNP mutations (Markussen et al., 2003). Interestingly studies on VKOR activity have demonstrated that several mutations to the *vkorc1* gene result in lower VKOR activity (i.e. V_{max} and K_m) indicating a trade-off between resistance and vitamin K cycling efficiency (Berny, 2011; Lasseur et al., 2006 & 2007). Debaux et al. (2019) have recently demonstrated that several known AVK resistance SNPs located on the *vkorc1 gene* affecting the VKOR codons 120 and 139 severely reduced vitamin K cycling efficiency in rats, further supporting the concept that AVK resistance comes with an associated biological cost to vitamin K metabolism.

Reproduction in bromadiolone-resistant *R. norvegicus* strains has also been reported to be impaired. The study of Heiberg et al. (2006) found moderately resistant females to have higher reproductive success than highly resistant females: however, among males, the opposite was true. This body of research indicates that there are some clear biological costs associated with AVK resistance in *R. norvegicus*, but little information is available regarding other rodent species.

5. Techniques for determining resistance

Several experimental methods have been developed for identifying AVK resistance in rodent species. However, each test has varied in their respective strengths and weaknesses e.g. accuracy, costs, difficulty and duration. While cage feeding trials have been the longest established experimental method for determining AVK resistance, recent developments have aimed at producing more humane techniques of assaying resistance in rodents. Several techniques have been developed which measure functions of the blood clotting process. These tests have included measuring VKOR enzyme activity in liver microsome preparations or expression of recombinant *vkorc1* genes in cell lines and blood clotting responses (BCR) (Fasco et al., 1983; Gill et al., 1994; Hodroge et al., 2011; Pelz and Prescott, 2015). The review chapter of Berny et al. (2018) also covered these mechanisms recently. A brief overview of each method is given in the following sections of this manuscript for completeness.

5.1. Lethal feeding period test

The earliest rodenticide resistance tests relied on no choice feeding trials of caged rodent species where resistance was measured as a function of survival per quantity of AVK consumption (Berny et al., 2018). The earliest lethal feeding period (LPF) tests, such as the WHO LPF test, were no-choice trials conducted on rodents only provided with bait containing the rodenticide compound (Buckle et al., 1994; Garg and Singla, 2014[b]). As an experimental method the strongest point in favour of feeding trials is that they very reliably determine if resistance is present in the target rodent species. However, feeding trials require the capture, storage and handling of live wild rodents suspected of being resistant, this makes this technique resource intensive, time consuming and costly. Feeding trials generally range from 4 to 21 days which requires constant monitoring and recording of data by trained staff (Berny, 2011). In order to provide statistically robust data, feeding trials also require the capture of many live rodents from a population suspected of being resistant. This can often be challenging, and the number of rodents caught may not provide representative results.

5.2. Blood clotting response (BCR)

The BCR test was developed in the late 1970s with the aim of determining AVK resistance as a measure of the blood clotting activity in live rodents (Martin et al., 1979). The BCR test is similar to the feeding trials in that it requires rodents to be captured and caged. However, rather than feeding rodents with AVK bait they are ideally injected with a sub-lethal dose of AVK (this is to overcome issues of variable intestinal absorption), blood samples are subsequently taken and tested 1–4 days later to determine blood clotting activity (Gill et al., 1994; Pelz et al., 2005). The level of blood clotting activity is assessed and used as an end point for determining the level of any AVK resistance (Garg and Singla, 2015). The BCR test methodology removes the need to inflict mortality by poisoning on rodent test subjects and is considered more humane than feeding trials inducing mortality. There are some draw backs to the BCR test in that it is nearly as resource intensive as caged feeding trials and results have not been considered very precise being on occasion prone to producing false positives (Berny, 2011; Kerins and MacNicoll, 1999; Pelz et al., 2005). However, the test has been revised several times with the aim of improving sensitivity and improving the comparability of results (Prescott et al., 2007; Pelz and Prescott, 2015). Blood clotting response tests are particularly useful in that they provide a direct quantitative measure of physiological resistance, which captures resistance mediated by either pharmacokinetic measures or genetic mutations (Prescott et al., 2007). The Rodenticide Resistance Action Group (RRAC) website provides a list of BCR-derived AVK resistance ratios for a range of *R. norvegicus vkorc1* mutations and the Tyr139Cys *vkorc1* mutation in *M. musculus* (RRAC, 2016). These are useful for understanding the level of practical resistance conferred by each mutation and contrasting their potency.

5.3. Kinetic studies on VKOR activity

AVK resistance can also been directly assessed by measuring liver VKOR protein activity *in vitro* (Lasseur et al., 2006 & 2007; Goulois et al., 2017). This technique involves harvesting fresh rodent liver cells and producing a microsome preparation via centrifugation or, alternatively, rodent VKOR proteins can be produced using recombinant genes expressed in human embryonic kidney (HEK), the yeast *Pichia pastoris* or other suitable cell lines (Hodroge et al., 2011; Moroni et al., 1995; Rost et al., 2009). The final preparations obtained from these methods contain the VKOR protein which acts as the catalyst in reducing vitamin K epoxide into Vitamin K hydroquinone, the precursor required for production of blood clotting factors II, VII, IX, X (Fasco et al., 1983; Hadler and Buckle, 1992; Lasseur et al., 2006 & 2007). By measuring the kinetics of this VKOR reaction in the presence and absence of differing concentrations of AVKs, a level of resistance can be determined by comparing AVK-susceptible rodents to rodents with suspected resistance. However, in some cases discrepancies have been observed between rodent *in vitro* VKOR activity and observed *in vivo* resistance (Takeda et al., 2018). The Takeda et al. (2018) study proposed measuring pharmacokinetic clearance of AVKs *in situ* in rodents via liver perfusion activity, which may more accurately

Table 1
List of known resistance SNP mutations in the *R. norvegicus vkorc1* gene, the AVKs to which they confer resistance to if any, and the geographical locations where they have been identified in wild populations.

SNP	FGAR	SGAR	Location	Ref
Val12Leu			Azores	Rost et al. (2009)
Ala21Thr			Korea	Rost et al. (2009)
Ala26Thr			U.K.	Rost et al. (2009)
Arg33Pro			U.K.	Rost et al. (2009)
Arg35Pro			France; U.S.A.	Pelz et al. (2005); RRAC (2019)
Tyr39Asn			U.K.	Rost et al. (2009)
Ser56Pro			Germany	Pelz et al. (2005)
Trp59Arg			Argentina	Rost et al. (2009)
Phe63Cys			U.K.	Rost et al. (2009)
Glu67Lys			Japan	Rost et al. (2009)
Ile90Leu			Argentina; Azores; Indonesia; U.S.A.	Rost et al. (2009)
Leu120Gln	Chlorophacinone; Warfarin	Bromadiolone; Difenacoum	France; Netherlands; U.K.	Boitet et al. (2018); Hodroge et al. (2011); Grandemange et al. (2010); Pelz et al. (2005); RRAC (2019); Vein et al. (2013)
Ile123Ser			Italy	Iacucci et al. (2018)
Leu128Gln	Chlorophacinone; Coumatetralyl; Warfarin		France; U.K.	Grandemange et al. (2010); Hodroge et al. (2011); Pelz et al. (2005); Rost et al. (2009); RRAC (2019)
Leu128Ser			France	Grandemange et al. (2010)
Tyr139Cys	Chlorophacinone; Coumatetralyl; Warfarin	Bromadiolone; Difenacoum	Denmark; France; Germany; Hungry; Netherlands; U.K.	Grandemange et al. (2010); Hodroge et al. (2011); Pelz et al. (2005) & (2007); Markussen et al. (2008); Meerburg et al. (2014); Rost et al. (2009); RRAC (2019); Vein et al. (2013)
Tyr139Ser	Chlorophacinone; Coumatetralyl; Warfarin		U.K.	Hodroge et al. (2011); Rost et al. (2009)
Tyr139Phe	Chlorophacinone; Coumatetralyl; Warfarin	Bromadiolone; Difenacoum	Belgium; France; Korea; Netherlands; U.K.	Desvars-Larrive et al., (2017); Grandemange et al. (2009) & (2010); Hodroge et al. (2011); Lasseur et al. (2005); Meerburg et al. (2014); Pelz et al. (2005); Prescott et al., 2011 Rost et al. (2009); RRAC (2019); Vein et al. (2013)
Ile141Val			Indonesia	Rost et al. (2009)
Ala143Val			Indonesia; Thailand	Rost et al. (2009)

reflect observed *in vivo* activity.

5.4. Molecular characterisation of resistance – vkorc1 gene

A number of AVK resistance mechanisms have been proposed throughout the years, however, it is the role of the *vkorc1* gene that remains the most widely understood. The *vkorc1* gene was identified first in the human genome as a source of warfarin resistance by Rost et al. (2004). The gene was subsequently found to be homologous in rodent species playing a similar role in Vitamin K cycling and AVK resistance (Pelz et al., 2005). Certain SNP mutations in the *vkorc1* gene have been shown to produce translational differences in the VKOR protein amino acid sequence which reduce the ability of warfarin to inhibit the VKOR enzyme's activity. By reducing the ability of warfarin to bind the VKOR protein (biochemical kinetic studies have established that warfarin acts as a non-competitive inhibitor of the VKOR enzyme) AVK resistance is conferred in rodent species (Lasseur et al., 2006).

Since the discovery of the *vkorc1* gene's role in conferring AVK resistance in rodents, much attention has been paid to identifying sequence mutations and characterising their effect on AVK efficacy. Whether an individual rodent's *vkorc1* gene contains particular SNP mutations (Grandemange et al., 2009) and whether the rodent is homogenic or heterogenic for these mutations (Goulois et al., 2016) appears to confer varying degrees of resistance to certain FGAR and SGAR compounds. Several mutations of the *vkorc1* gene have been studied for conferring degrees of AVK resistance or reducing VKOR activity in *R. norvegicus*, *R. rattus* and *M. musculus* and are listed in Tables 1–3, respectively.

There are some limitations to using the *vkorc1* gene for characterising wild populations for AVK resistance. To date, many of the recent studies on *vkorc1* genes in wild rodent populations have characterised the presence/absence of *vkorc1* mutations in specific countries correlating these with observed resistance, however other mechanisms such as pharmacokinetics are not always investigated. Additionally, many studies have focused on determining VKOR biochemical kinetic responses to AVK compounds, though very few publicly available studies have been performed to date on resistant live rodent populations (Hodroge et al., 2011). The lack of *in vivo* studies directly investigating each possible mechanism of resistance in wild live resistant rodent populations is a particular limitation when trying to determine whether the *vkorc1* gene is the only basis of a particular observance of rodenticide resistance. Characterisation of *vkorc1* gene mutations does not reveal the full extent of AVK resistance in rodents.

As a technique, molecular characterisation of resistance has several distinct advantages over other methods of determining resistance. Molecular techniques are relatively rapid, high throughput and relatively cheap compared to alternative methods. For molecular characterisation of the *vkorc1* gene rodents need not be captured alive, recently deceased subjects can be characterised by simply removing the end portion of the tail (2–3 cm) and storing this for molecular characterisation. Additionally, rodent DNA can be obtained from faecal pellets/scats for *vkorc1* characterisation allowing for monitoring of wild uncaptured populations, though failure rates for PCR performed on DNA obtained from scats have been reported to be higher than those performed on tails (Pelz, 2007; Meerburg et al., 2014). However, when collecting scat

Table 2
List of known resistance SNP mutations in the *R. rattus vkorc1* gene, the AVKs to which they confer resistance to if any, and the geographical locations where they have been identified in wild populations.

SNP	FGAR	SGAR	Location	Ref
Ala14Val			New Zealand	Cowan *et al.*, (2016);
Tyr25Phe		Bromadiolone; difenacoum; difethialone	Spain; New Zealand	Cowan et al. (2017); Goulois et al. (2016)
Ala26Val			New Zealand	Cowan et al., (2017)
Ala41Thr	Warfarin		Japan	Tanaka et al. (2012)
Ala41Val	Warfarin		Japan	Tanaka et al. (2012)
Arg61Trp	Warfarin		Japan	Tanaka et al. (2012)
Leu76Pro	Warfarin		Japan	Takeda et al. (2016); Tanaka et al. (2012)
Ile90Leu			New Zealand	Cowan et al., (2017)

Table 3
List of known resistance SNP mutations in the *M. musculus vkorc1* gene, the AVKs to which they confer resistance to if any, and the geographical locations where they have been identified in wild populations.

SNP	FGAR	SGAR	Location	Ref
Arg12Trp			France; Germany	Rost et al. (2009); Goulois et al. (2017b)
Ala21Thr		Bromadiolone	Serbia	Scepovic et al. (2016)
Ala26Ser			France	Goulois et al. (2017b)
Ala26Thr		Bromadiolone; Difenacoum	France	Goulois et al. (2017b)
Arg35Pro			U.S.A.	RRAC et al. (2019)
Glu37Gly			France	Rost et al. (2009);
Ala48Thr			France	Goulois et al. (2017b)
Arg58Gly			France	Endepols et al. (2013); Goulois et al. (2017b)
Trp59Gly	Warfarin		France; Germany	Lasseur et al. (2006); Goulois et al. (2017b); Rost et al. (2009)
Arg61Leu			France	Goulois et al. (2017b)
Leu124Met			France	Goulois et al. (2017b)
Leu128Ser	Warfarin; Coumatetralyl; Chlorophacinone	Bromadiolone; Difethialone; Brodifacoum	Azores; France; Germany; Ireland; Serbia; Switzerland; U.K.	Goulois et al. (2017b); Mooney et al. (2018); Pelz et al. (2005); Rost et al. (2009); RRAC (2019); Scepovic et al. (2016);
Tyr139Cys	Warfarin; Coumatetralyl; Chlorophacinone	Bromadiolone	Azores; France; Germany; Ireland; Serbia; Switzerland; U.K.	Blazic et al. (2019); Goulois et al. (2017b); Mooney et al. (2018); Pelz et al. (2005); Rost et al. (2009); RRAC (2019); Scepovic et al. (2016);
Arg12Trp, Ala26Ser, Ala48Thr and Arg61Leu (*vkorc1*^{spr})	Coumatetralyl; Chlorophacinone	Bromadiolone; Difenacoum	France; Germany; Spain; Switzerland	Goulois et al. (2017a) and (2017[b]); RRAC (2019); Song et al. (2011)
Ala26Thr/Leu128Ser	Coumatetralyl; Chlorophacinone	Brodifacoum; Bromadiolone; Difenacoum; Difethialone	France	Goulois et al. (2017b)
Ala26Ser/Leu128Ser	Coumatetralyl; Chlorophacinone	Brodifacoum; Bromadiolone; Difenacoum; Difethialone	France	Goulois et al. (2017b)
Trp59Gly/Leu124Met[a]			France	Goulois et al. (2017b)
Trp59Gly/Leu128Ser[a]			France	Goulois et al. (2017b)
Leu128Ser/Tyr139Cys		Bromadiolone	Serbia	Blazic et al. (2019)

[a] Resistance undeterminable as mutant VKOR enzyme activity *in vitro* <2% of wild type making assessment untestable.

samples from a particular area, it cannot be determined if several collected samples belong to the same individual rodent or several individuals.

6. Known resistance mechanisms in rodent species

6.1. Rattus norvegicus

Rattus norvegicus, also known as the “Norway”, “brown” or “common rat”, is a rodent species which is suspected to have originated in the cold northern climates of China and Mongolia prior to becoming a major worldwide invasive species (Puckett et al., 2016). In contrast to the black rat (*R. rattus*) and the house mouse (*M. musculus*) whose historical distribution closely matches human regional agricultural development, the global expansion of the brown rat is a relatively recent event with populations only establishing in Europe and America in the 1500s and 1750s, respectively (Puckett et al., 2016). *Rattus norvegicus* populations have been shown to be highly adaptable in altering their life-cycle depending on the type of environment they inhabit. Vadell et al. (2014) identified life-cycle differences between *R. norvegicus* populations inhabiting urban and rural/sylvan settings. Reproduction was reported to be generally highest in urban centres during Spring and Autumn, while rural populations tend to reproduce more during Winter (Vadell et al., 2014). However, annual variations in reproduction rates reflecting the degree of exposure populations had to seasonal weather variations, such as sub-zero temperatures and frost, was also highlighted as an influential factor affecting population dynamics. This was most evident in sylvan populations, which were the most exposed to seasonal weather patterns. Due to its highly adaptable nature, this species is now commonly found inhabiting urban environments alongside human populations (Desvars-Larrive et al., 2017). This close proximity to human settlements has resulted in several problems becoming associated with their presence, such as their capacity to spread zoonotic diseases to humans, companion animals and farm stock and to inflict

damage to property (Desvars-Larrive et al., 2017; Meerburg et al., 2009). Control of this invasive species has now become critical in urban centres across the world and the increasing frequency of reported AVK resistance in wild populations poses a serious threat to maintaining public health.

The capability of *R. norvegicus* populations to develop resistance to AVK compounds has long been documented. Soon after the introduction of AVK rodenticides in the early 1950s, warfarin and diphacinone resistance was detected in a *R. norvegicus* population in Scotland in 1958 (Boyle, 1960). Some studies have indicated that resistance factors were already present in several UK *R. norvegicus* populations prior to the use of AVKs and have since become more prevalent due to the selective pressure exerted from AVK use (Greaves and Rennison, 1973; Buckle, 2013). A clear inheritable genetic component to *R. norvegicus* AVK resistance was identified in 1967 and by 1973 it was known that the vitamin K cycling VKOR enzyme was the target of AVKs, however, the identity of the resistance genes responsible remained unknown (Greaves and Ayres, 1967; Whitlon et al., 1978). It was not until the 21st century that the *vkorc1*-mediated genetic basis of resistance to AVK compounds was established by Rost et al. (2004), their study identified specific mutations which conferred resistance to warfarin in human patients. The work of Pelz et al. (2005) subsequently demonstrated that AVK resistance in *R. norvegicus* could also be mediated by mutations in the *vkorc1* gene. The Pelz et al. (2005) study identified 7 mutations (Arg35Pro, Ser56Pro, Leu120Gln, Leu128Gln, Tyr139Ser, Tyr139Cys and Tyr139Phe) of the *R. norvegicus vkorc1* gene which resulted in changes in the amino acid sequence conferring resistance to warfarin. The study also demonstrated that mutations which conferred warfarin resistance such as Leu120Gln, Leu128Gln, Tyr139Cys and Tyr139Ser resulted in dramatically reduced basal activity of the VKOR enzyme. However, despite the lower level of basal VKOR enzyme activity in the mutated *vkorc1* genes, each mutation was found to confer greater resilience in the presence of various concentrations of warfarin compared to the wild-type *vkorc1* gene. This resilience was identified as the physiological mechanism by which they conferred AVK resistance. The Pelz et al. (2005) study demonstrated that the physiological cost for *vkorc1* SNP mediated AVK resistance could possibly be a lower basal activity of the VKOR enzyme. The study of Lasseur et al. (2005) confirmed that the Tyr139Phe mutation of the *R. norvegicus vkorc1* gene conferred resistance to warfarin via greater resilience in VKOR enzyme activity. However, Lasseur et al. (2005) also found the Tyr139Phe mutation to lower basal VKOR enzyme activity compared to an unmutated wild-type VKOR, this was in direct contrast to the findings of Pelz et al. (2005) which found activity to be increased for this particular mutation. Regardless of the discrepancy between the studies, both studies support a general association of AVK resistance coming with a physiological trade-off in *R. norvegicus* i.e. impaired vitamin K metabolism.

To date, several identified *vkorc1* SNPs have been tested individually for their capacity to confer AVK resistance. However, there is limited data on the effects of combinations of different mutations on resistance. This has resulted in an incomplete picture of *vkorc1* SNP mediated resistance in *R. norvegicus*. Further complicating matters is the tendency of many surveys to only report the presence or absence of *vkorc1* SNP mutations identified in wild populations and not test the actual physiological level of resistance, thus overlooking possible pharmacokinetic resistance factors that may be present. Pelz (2007) recommended that PCR techniques should be used to survey for the presence of known *vkorc1* AVK resistance SNP mutations in *R. norvegicus* populations and that the degree of resistance to more potent compounds should be determined using BCR tests on live caught individuals. The study of Grandemange et al. (2009) indicated that individual *vkorc1* mutations could confer resistance to a broad range of AVK compounds. In their study, it was demonstrated that resistance to chlorophacinone, bromadiolone and low doses of difenacoum could be mediated by the Tyr139Phe SNP mutation alone. However, their study indicated that resistance to more potent SGARs such as difethialone and high doses of difenacoum would require additional mutations to the *vkorc1* gene and/or other genes/pharmacokinetic mechanisms.

The ability of a single SNP mutation to confer tolerance to a broad range of AVKs compounds, given their same site of action, explains the commonly observed phenomena of cross-resistance (e.g. situations where resistance to one AVK compound appears to confer resistance to another) in *R. norvegicus* populations. Several studies have identified instances of the development of cross-resistance within wild *R. norvegicus* populations. The BCR test was used for determining the degree of resistance for and cross-resistance to AVK compounds (coumatetralyl and bromadiolone) in wild *R. norvegicus* populations in Germany and the UK by Endepols et al. (2007). Similarly, using the BCR test, Buckle et al. (2007) identified a degree of cross-resistance to bromadiolone in Welsh *R. norvegicus* populations known to be resistant to warfarin. However, while a degree of bromadiolone resistance was observed, the infestation was controllable with a targeted 35-day bromadiolone baiting approach, though notably females were identified as being more resistant than their male counterparts. Resistance to the SGAR, bromadiolone appears to be highly cross-linked to FGAR resistance, however, resistance to bromadiolone int rats has been shown to be breakable using a combination of the FGAR coumatetralyl and an enhancer such as cholecalciferol (Endepols et al., 2017). The RRAC (2016) report indicated that in *R. norvegicus* the Leu120Gln *vkorc1* substitution mutation conferred the broadest degree of AVK resistance (bromadiolone, difenacoum, brodifacoum, flocoumafen, difethialone) of mutations currently characterised. The RRAC (2016) report revealed that the Tyr139Ser and Tyr139Phe mutations both conferred a high level of resistance to bromadiolone and similar low levels of resistance to the other SGARs.

Several investigations into the geographical distributions of the *vkorc1* gene mutations in *R. norvegicus* populations have been conducted to date and the resistance conferred by certain mutations has been characterised (see for example in UK RRAG, 2018). A list of all currently identified *R. norvegicus vkorc1* gene SNP mutations, their global distribution and the AVKs to which they have been seen to confer resistance is presented in Table 1. The Pelz et al. (2005) study identified the Tyr139Cys *vkorc1* mutation in wild rodents in a German region and in their follow up study they established that this mutation was in fact widespread across western Germany (Pelz, 2007). In a wide-ranging study investigating *R. norvegicus* samples obtained from four continents, Rost et al. (2009) established that the *vkorc1* gene was the site of spontaneous mutations conferring warfarin resistance. The Rost et al. (2009) study identified 25 *vkorc1* mutations, eight of which possessed a proven ability to confer resistance to warfarin. Though the majority of the mutations were not found to confer substantial resistance when assessed in BCR or feeding trials these mutations were shown to tolerate AVKs to varying degrees. Grandemange et al. (2010) detected a range of *vkorc1* mutations (Ser103Tyr, Leu120Gln, Leu128Gln, Leu128Ser, Glu155Lys, Tyr139Phe and Tyr139Cys) in French *R. norvegicus* populations suspected of being AVK-resistant. Their study revealed that the Tyr139Phe mutation was spreading rapidly throughout France and was present in 28% of *R. norvegicus* samples. This work was supported by the study of Desvars-Larrive et al. (2017) which also found a high prevalence of the Tyr139Phe *vkorc1* mutation in a French urban environment. The Tyr139Phe and Tyr139Cys mutations have also been shown to be abundant in the Netherlands (Meerburg et al., 2014). The Prescott

et al., 2011 study first reported that the Tyr139Phe mutation was present in the UK and suggested that it would be prudent to halt the use of FGARs and bromadiolone in instances where this mutation was identified in *R. norvegicus* populations. The mutations Leu120Gln, Tyr139Cys and Tyr139Phe were postulated to be the most potent mutations conferring AVK resistance in UK *R. norvegicus* populations which threatened effective rodent control (Buckle, 2013; Prescott et al., 2011: Jones et al., 2019). Hodroge et al. (2011) demonstrated *in vitro* that the mutations Leu120Gln, Leu128Gln, Tyr139Cys, Tyr139Phe and Tyr139Ser conferred significant resistance to certain FGARs (warfarin and chlorophacinone) and to a lesser extent the SGAR, bromadiolone. However, the Hodroge et al. (2011) study found the Leu120Gln mutation to confer the greatest degree of resilience to SGARs brodifacoum, bromadiolone, difenacoum and difethialone compared to the Leu128Gln and the Tyr139 mutations Cys, Phe and Ser. This was supported in *in vivo* feeding trials investigating resistance to the FGAR, chlorophacinone in resistant *R. norvegicus* samples in France, where it was demonstrated that rats possessing the Leu120Gln mutation had greater survival times than rats possessing either of the Tyr139Phe or Tyr139Cys mutations (Vein et al., 2013). The potency of the Leu120Gln mutation was further demonstrated by the study of Boitet et al. (2018) in which a rat strain homozygous for the Leu120Gln mutation was shown to be severely resistant to the SGAR, difenacoum. Other mutations of the *vkorc1* gene have been reported such as Ile123Ser mutation in Italian *R. norvegicus* populations but have not yet been investigated for the level of conferred resistance (Iacucci et al., 2018). However, a similar mutation in humans (Ile123Asn) is known to confer warfarin resistance (Boitet et al., 2018). The Boitet et al. (2018) study also identified that enhanced anticoagulant resistance relied on enhanced cytochrome P-450 oxidative metabolism in addition to the Leu120Gln mutation, supporting the Markussen et al. (2008) study which indicated that SGAR resistance could have a multifactorial pharmacokinetic dimension. Few surveys of AVK resistance monitoring in countries are currently available but a report by Jones et al. (2019) on *vkorc1* mutations in *R. norvegicus* populations in the UK indicated that up to 60% of samples contained at least one known resistance mutation.

As previously alluded too, a possible pharmacological mechanism of AVK resistance has also been proposed for *R. norvegicus*. A study by Lasseur et al. (2007) suggested that the resilience in VKOR enzyme activity in Tyr139Phe mutant *R. norvegicus* was not sufficient alone to explain warfarin resistance in a French strain. Their investigation into the biochemistry of warfarin resistance revealed that the enzymatic efficiency (V_{max}/K_m) of VKOR between wild-type VKOR and Tyr139Phe VKOR was similar, indicating the possibility of other mechanisms mediating AVK resistance. The Markussen et al. (2008) study supported the idea that AVK resistance could have a pharmacokinetic component when they demonstrated that cytochrome P-450 activity was increased in bromadiolone-resistant male and female *R. norvegicus*. The cytochrome P-450 family is known to be involved in metabolising potentially toxic compounds. Their study also indicated a gender resistance factor with females over expressing the Cyp2e1 P-450 gene, possibly explaining why females are reported to be more resistant than males in many studies.

Female *R. norvegicus* have been observed to display greater AVK resistance than males in many studies. However, it is only recently that light has been shed on the possible mechanism for increased resistance in female *R. norvegicus*. This topic was explored in the study of Lefebvre et al. (2016) where the role of gender was demonstrated to play a significant pharmacokinetic role in *R. norvegicus* AVK resistance. The Lefebvre et al. (2016) study found that certain blood clotting factors had higher basal activity (VII and X) and longer half-lives (II and X) in females compared to males after administration of difethialone. The resulting greater resilience in blood clotting activity offers an explanation for the reduced efficacy of AVK compounds observed in female *R. norvegicus* in many other studies such as Boitet et al. (2018); Endepols et al. (2017) and Markussen et al. (2008).

In summary, while there are still some gaps in the understanding of AVK resistance in *R. norvegicus* there are several clear patterns emerging from the current body of research. Female *R. norvegicus* are inherently more resilient to AVK poisoning than males, and the mechanism of resistance appears to be pharmacokinetic, this point is particularly supported by the research of Lefebrve et al. (2016) and Markussen et al. (2008). Additional pharmacokinetic-mediated AVK resistance mechanisms, such as enhanced cytochrome P-450 activity, may play a role in resistance to both FGAR and SGAR AVK compounds in both males and females. AVK resistance to FGARs in *R. norvegicus* populations is becoming widespread and appears to afford a degree of cross-resistance to the SGAR bromadiolone. The spread of resistance appears to be particularly linked to SNPs in the *vkorc1* gene which confer varying degrees of resistance to FGARs and SGARs. SNP mutations affecting the tyrosine amino acid at position 139 appear to confer significant FGAR resistance and limited SGAR resistance. SNP mutations affecting Leu120 seem to confer significant FGAR and SGAR resistance making the spread of this mutation particularly concerning, particularly when paired with an additional SNP mutation of the Tyr139 codon. However, there are still countries with a long history of AVK use where *vkorc1* mutations conferring resistance in wild *R. norvegicus* populations has not as of yet been identified such as Ireland and New Zealand, though this may be a result of the limited sampling carried out in these surveys (Cowan et al. 2017; Mooney et al. 2018). However, certain silent *vkorc1* mutations have been observed in *R. norvegicus* populations in several countries which result in no change in the amino acid sequence. While these silent mutations, such as at codon 82, do not affect VKOR activity, they have been hypothesised to reduce the transcriptional activity of the *vkorc1* gene, therefore having an indirect effect on resistance (Mooney et al., 2018).

6.2. *Rattus rattus*

Rattus rattus (also known as the “black rat”, “house rat”, “ship rat” or “roof rat”) is a rodent species that originated in Asia and has subsequently spread to inhabit every continent with the only known exception being Antarctica (Aplin et al., 2011). *Rattus rattus* is regarded as a highly invasive species and can be extremely destructive when established in new environments. Invasive *R. rattus* have been shown to destabilise native plant populations through the removal and consumption of seeds in South American rain forest floors (Shiels and de Arellano, 2019). Establishment of this species in new locations, particularly islands, has also led to serious threats to indigenous wildlife populations (Wheeler et al., 2019). This rodent is also a significant pest in urban and agricultural settings, while additionally being a vector of numerous zoonotic diseases such as the plague-causing bacterium *Yersinia pestis* (Aplin et al., 2011). As a result of the ubiquitous distribution of *R. rattus* and its enormous negative economic impacts there have been many costly efforts to control this species. Despite this, rodenticide resistance in wild populations of the *R. rattus* has been poorly characterised compared to other rodent species (Goulois et al., 2016). Early rodenticide research demonstrated that AVK resistance could develop in *R. rattus* similarly as in other rodent species. Greaves et al. (1976a) observed exceptional levels of warfarin resistance in wild populations of *R. rattus* in the UK in the 1970s. However, in a follow up study Greaves et al. (1976b) found

the resistance mechanism to be unstable with less than 1% of offspring retaining resistance. Greaves et al. (1976b) proposed that a multi-factorial form of warfarin resistance was present in crossbred strains of *R. rattus* based on an observed instability of inherited resistance.

Resistance in wild populations of *R. rattus* appears to be widespread in certain areas; in North Eastern Germany between 1980 and 1990 warfarin resistance was shown to have developed in 6 out of the 7 surveyed populations (Endepols et al., 1991). The results from an EPPO questionnaire on AVK resistance in wild rodents disseminated in 1992 revealed that warfarin-resistant *R. rattus* populations were present in many countries including Denmark, France, Germany and the UK (Myllymaki, 1995). The EPPO questionnaire also revealed that *R. rattus* in France had been documented to be resistant to both diphacinone and bromadiolone (Myllymaki, 1995).

The current literature suggests that AVK resistance in *R. rattus* is a result of a several different mechanisms. Behavioural resistance in the form of bait avoidance has been observed in *R. rattus* populations in animal housing (Leung and Clark, 2005). *Rattus rattus* have also been shown to be very particular regarding their acceptance of different types of bait taste additives (Shafi et al., 1990). Interestingly, there have also been numerous studies demonstrating a metabolic resistance to AVKs. The studies undertaken by Sugano et al. (2001) and Ishizuka et al. (2007) indicated that warfarin resistance in *R. rattus* populations in Tokyo was facilitated by elevated activity of certain cytochrome P-450 subfamilies which resulted in high levels of metabolic clearance. Warfarin metabolism has been shown to be mainly regulated by the CYP1A, CYP2B, CYP2C and CYP3A subfamilies (Takeda et al., 2016). Cytochrome P-450-mediated warfarin resistance was also detected in Tokyo *R. rattus* by Takeda et al. (2016) with CYP2B, CYP2C and CYP3A highly expressed in resistant rats. However resistant rats also possessed an SNP mutation in the *vkorc1* gene which resulted in the Leu76Pro substitution in the VKOR protein. The Leu76Pro mutation had been shown to confer warfarin resistance in wild populations of Japanese *R. rattus* (Tanaka et al., 2012), in addition to several other mutations conferring warfarin resistance such as Ala41Thr, Ala41Val and Arg61Trp. A full list of the currently known SNP mutations of the *R. rattus vkorc1* gene is provided in Table 2 alongside the AVK compounds to which they provide resistance and in which countries these SNP mutations have been detected. A pharmacokinetically-mediated resistance to warfarin in *R. rattus* was further supported by the study of Takeda et al. (2018) in which they also developed a novel assay for measuring warfarin metabolism *in situ*. In the Takeda et al. (2018) study, expression of certain P-450 cytochromes such as CYP1A, CYP2B1, CYP1C11 and CYP3A2 were significantly higher in resistant rats compared to susceptible rats. Their study also investigated the often-reported observed discrepancies between *in vivo* warfarin tolerance of *R. rattus* and the *in vitro* measured VKOR activity, their novel technique for measuring hepatic warfarin metabolic activity in a liver perfusion was found to more accurately reflect the level of observed resistance in *R. rattus*. The current body of research shows that resistance to the FGAR warfarin in Japanese *R. rattus* wild populations is strongly mediated by pharmacokinetic mechanisms, though there also appears to be a *vkorc1* genetic component in some cases.

Characterisation of 153 individuals of *R. rattus* obtained from 9 Bulgarian farms identified both FGAR- and SGAR-resistant populations in Europe (Zhelev et al., 2019). This study identified that of the *R. rattus* samples, 92.1% were warfarin resistant, while 62.5% were also resistant to coumatetralyl. The Zhelev et al. (2019) study also characterised SGAR resistance finding that 38.5% of the *R. rattus* population was resistant to bromadiolone, though none was found to be resistant to brodifacoum. However, the underlying mechanism of resistance in the Bulgarian population was not established. The first described *vkorc1*-mediated mechanism of AVK resistance present in wild *R. rattus* European populations was identified in Spain in 2016 (Goulois et al., 2016). Goulois et al. (2016) attributed resistance to a novel mutation on the *vkorc1* gene (Tyr25Phe) which *in vitro* experiments demonstrated conferred differing degrees of resistance to warfarin, bromadiolone, difenacoum and difethialone. The Tyr25Phe mutation was also detected in a survey *vkorc1* genes in *R. rattus* populations in New Zealand, along with several other mutations Ala14Val, Ala26Val and Ile90-Leu (Cowan et al., 2017). Cowan et al. (2017) did not establish if these mutations conferred resistance to AVK compounds but suspected possible resistance based on the study of Goulois et al. (2016).

Bromadiolone-resistant populations of *R. rattus* have also been identified in India (Garg and Singla 2014a, 2015; Garg et al., 2017), where wild rats were confirmed to be resistant using a combination of feeding trials and BCR tests. Using feeding trials, Garg and Singla (2014a) found bromadiolone resistance to be inherently more prominent in females compared to males in an Indian population of *R. rattus* obtained from poultry farms. A standardised BCR test for determining bromadiolone resistance based on prothrombin time in *R. rattus* was developed by Garg and Singla (2015). Molecular characterisation was done for confirming suspected bromadiolone resistance in wild Indian *R. rattus* populations by Garg et al. (2017). However, no known SNP resistance mutations were identified in the sequenced *vkorc1* genes, indicating a probable pharmacokinetic form of bromadiolone resistance in the Indian populations (Garg et al., 2017).

Whether female *R. rattus* are inherently more AVK resistant than their male counterparts has not yet been extensively explored but it appears gender may play a role based on the study conducted by Garg and Singla (2014a). To date it has also been demonstrated that female *R. rattus* are more tolerant of other non-AVK rodenticides, such as cholecalciferol (Vitamin D_3), than males (Kaur et al., 2008). *Rattus rattus* has also been shown to be more sensitive to cholecalciferol than other rodent species such as *R. norvegicus*, particularly when it is used in combination with warfarin (Bai et al., 1978). Similarly, Kocher and Harjot (2013) demonstrated that using cholecalciferol synergistically with bromadiolone produced enhanced efficacy against *R. rattus,* though no difference was observed between the genders. Behaviourally, *R. rattus* females are known to range over far smaller areas than males in the wild and that this behaviour is not altered when poisoned with AVKs such as brodifacoum (Hooker and Innes, 1994). One method of directly controlling *R. rattus* by gender was reported by Siers et al. (2017) when a wild Hawaiian population was administered bait containing two non-toxic fertility controlling chemicals which successfully impaired spermatogenesis in males and ovulation in females leading to a reduction in litter production. However, use of gender-targeted measures remains limited to date and the reasons why chemical control using either AVKs or cholecalciferol are less effective at controlling *R. rattus* females than males is still currently little understood.

The limited research into AVK resistance in *R. rattus* has resulted in large gaps in understanding the mechanisms of resistance in wild populations. The presence or absence of resistance levels in wild populations in most countries is also currently unknown. It is clear from the literature that AVK resistance to both FGARs and the SGAR, bromadiolone in *R. rattus* is strongly linked to pharmacokinetic mechanisms such a P-450 cytochrome activity. As previously observed in other rodent species, SNP mutations affecting the *vkorc1* gene play a role in *R. rattus* AVK resistance but do not appear to be the dominant mechanism of resistance from observations to date. Many of the SNP AVK resistance mutations which have

evolved independently many times in *R. norvegicus* have not yet been observed in *R. rattus.* In order to more fully understand the mechanism of resistance in *R. rattus* further research is necessary to identify and characterise the levels of AVK tolerance present in wild populations of *R. rattus*. This will be critical in determining whether there is a potential risk of current AVK usage practices contributing to the development of resistance in wild populations.

6.3. *Mus musculus*

Mus musculus otherwise known as the house mouse, is a globally distributed commensal rodent which contains several recognised sub-species (Harr et al., 2016). The *M. musculus* sub-species complex includes *M. m domestics* (western house mouse), *M.m. musculus* (eastern house mouse) and *M.m. castaneus* (south-east Asian house mouse) all of which have their origins in a common ancestor originally located in southern Asia some 0.5 million years in the past (Harr et al., 2016). A closely related species to the *M. musculus* sub-species complex is the Algerian mouse *Mus spretus*, which diverged from *M. musculus* roughly 2 million years ago, though interestingly viable off-spring appear to have been produced from crosses between the two species at some point in the past (Harr et al., 2016). *Mus musculus* has now come to be one of the most widely distributed invasive species in the world and has even managed to establish populations on sub-Antarctic islands where the effect of their burrowing is causing massive environmental damage by destabilising surfaces (Eriksson and Eldridge, 2014). The introduction of *M. musculus* to many new locations has led to devastating impacts on a range of different organisms including plants, birds and vertebrates (Eriksson and Eldridge, 2014). Once established, *M. musculus* populations are very resilient, litter sizes appear to be rarely affected by varying seasonal environmental conditions, reproduction rates appear to peak during the summer months, the combination of these factors leads to highly abundant populations (Vadell et al., 2014). As a consequence, control of invasive *M. musculus* populations is now carried out throughout the world in the interests of both human hygiene and environmental protection. However, since the introduction of AVK chemical control measures for *M. musculus,* there have been growing reports of resistance in wild populations, some as early as the 1960s (Dodsworth, 1961; Rowe and Redfern, 1964). Resistance to AVK compounds is most frequently associated with FGARs such as warfarin but resistance to SGARs such as bromadiolone is also now frequently reported (Rowe and Redfern, 1964; Rowe et al., 1981; Leon et al., 2017). In order to safeguard the continued efficacy of existing AVKs, a greater understanding of the resistance mechanisms in *M. musculus* is necessary.

To date many studies have documented the distribution and extent of AVK resistance in *M. musculus* populations. These studies have revealed that several different AVK resistance mechanisms have developed in *M. musculus* populations throughout the world. These include hybridisation events between species introducing resistance mechanisms across a species barrier (Goulois et al., 2017a), spontaneous *de novo* genetic mutations of the *vkorc1* gene inducing VKOR enzyme resilience to AVKs (Lasseur et al., 2006), possible pharmacokinetic AVK resistance mechanisms (Endepols et al., 2013) and the role of genders' influence on AVK resistance (Scepovic et al., 2016). Attempts to map the global distribution of AVK resistance mutations have been limited to date. The RRAC (2019) provides a geographical map of countries where *vkorc1* SNP mutations have been identified in wild populations but this does not represent other resistance mechanisms. We have provided a summary of all the currently identified resistance mechanisms identified in both wild and laboratory strains of *M. musculus* below, along with studies investigating VKOR activity of known *vkorc1* mutations.

Similar, to rat species, AVKs enact their anticoagulant properties in house mice by targeting the activity of the VKOR enzyme. However, studies on VKOR activity in mice have indicated a complex relationship between warfarin resistance and VKOR activity. In a study conducted on known warfarin-resistant *M. m. musculus* captured in Denmark, two distinct responses were detected in VKOR activity in response to warfarin (Misenheimer et al., 1994). In the Danish study one warfarin-resistant group's VKOR activity was found to be insensitive to both warfarin and bromadiolone, conversely, VKOR activity in the second group of warfarin-resistant mice was found to be highly sensitive to warfarin. The Misenheimer et al. (1994) study indicated that two or more resistance mechanisms may have been present in different warfarin-resistant *M. musculus* populations in Denmark. The response of *M. m.* domesticus VKOR activity to warfarin was further characterised by Lasseur et al. (2006). The Lasseur et al. (2006) study found resilience of VKOR activity in a French strain of warfarin-resistant *M. musculus* to be attributed to the Trp59Gly mutation of the *vkorc1* gene. Their study hypothesised a bi-component model for VKOR activity which could be explained by either post-translational modifications to the VKOR protein or possibly separate isoforms of the *vkorc1* gene, though neither has yet been proven. VKOR activity in warfarin-resistant mice was found to be lower than VKOR activity in warfarin-susceptible mice indicating that while the mutation conferred the beneficial resistance trait it also impaired the functioning (V_{max}) of the enzyme, a possible biological cost to AVK resistance. The possibility of an alternative resistance mechanism to *vkorc1* mutations has also been supported by the study of Endepols et al. (2013) which investigated difenacoum-resistant mice obtained from 2 farms in Germany only identifying wild type *vkorc1* genes containing the Arg58Gly mutation. However, the Arg58Gly SNP was not found to correlate to difenacoum resistance. The geographic proximity of Denmark and Germany indicate the possibility of an as of yet uncharacterised mechanism of AVK resistance in *M. m. domesticus* populations in this region.

A study conducted by Song et al. (2011) demonstrated that resistance of certain populations of *M. musculus* in Europe was conferred through reproductive crosses with the Algerian mouse *M. spretus*. The Song study found that offspring from *M. m. domesticus* and *M. spretus* crosses had produced resistant *M. m. domesticus* populations which possessed complete or partial alleles of the *M. spretus vkorc1* gene (*vkorc1*^{spr}). The *vkorc1*^{spr} gene sequence is substantially different from the *M. m. domesticus vkorc1* (*vkorc1*^{dom}) producing a VKOR protein with a considerably different amino acid sequence. Mortality of mice homozygous for the *vkorc1*^{spr} gene when fed a diet of 375 ppm coumatetralyl or 50 ppm of either bromadiolone or difenacoum demonstrated greater resistance, particularly so in females, compared to their *vkorc1*^{dom} counterparts. The study of Goulois et al. (2017a) further characterised *vkorc1*^{spr} mediated AVK resistance in European *M. m. domesticus* populations. The study of Goulois et al. (2017a) compared male and female *M. m. domesticus* strains which were homozygous for either the *vkorc1*^{dom} or *vkorc1*^{spr} genes against a selection of FGARs (coumatetralyl and chlorophacinone) and SGARs (brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen). Their study identified four mutations (Arg12Trp, Ala26Ser, Ala48Thr and Arg61Leu) in the *M. m. domesticus vkorc1*^{spr} gene allele which appeared to confer resistance to the FGARs (coumatetralyl and chlorophacinone) and SGARs (bromadiolone and difenacoum) in feeding trials. However, no significant resistance was found to the SGARs brodifacoum, difethialone and flocoumafen. The four mutations appear to be a result of the introgression of the *M. spretus vkorc1* gene into the *M. m. domesticus*

population through hybridisation. The *vkorc1*^{spr} allele appears to have become firmly established in certain European populations and may possibly spread further in the future. Taken altogether, this body of work has demonstrated that hybridisation events could influence AVK resistance in other rodent species. The introduction of rodent species into new areas containing similar rodent species, previously separated geographically, may possibly result in hybridisation events, though no similar events have currently been reported.

Warfarin resistance conferred by a range of specific mutations in the *M. m. domesticus vkorc1* gene was characterised in the study of Rost et al. (2009). The Rost et al. (2009) study identified several mutations (Arg12Trp, Ala26Ser, Glu37Gly, Ala48Thr, Arg58Gly, Arg61Leu and Leu128Ser) in *M. m. domesticus* many of which reduced VKOR activity (with the exception of Ala48Thr, which increased VKOR activity), however, none conferred significant warfarin resistance *in vitro*. The exception was Tyr139Cys which both reduced VKOR activity and conferred a degree of resistance to warfarin. A list of currently identified *M. m. domestics vkorc1* SNPs mutations is presented in Table 3. The study of Endepols et al. (2013) also identified the Arg58Gly SNP mutation in populations of wild *M. m. domesticus* in Germany but found it uncorrelated to observed difenacoum resistance. Resistance to other SGARs has also been reported in wild *M. musculus* populations. Bromadiolone resistance has been identified in wild Argentinian *M. musculus* populations and the resistance mechanism was shown to be inheritable (Leon et al., 2017). However, the underlying mechanism of resistance was not identified in the Argentinian populations. Bromadiolone resistance has also been reported in *M. musculus* in Serbia but this was found to be associated with three *vkorc1* SNP resistance mutations Leu128Ser, Tyr139Cys and Ala21Thr (Scepovic et al., 2016). The Scepovic et al. (2016) study confirmed the findings of Rost et al. (2009) by demonstrating that VKOR activity was reduced by the Tyr139Cys and Leu128Ser mutations. A novel *vkorc1* SNP mutation Ala21Thr was identified in bromadiolone-resistant *M. musculus* in the Scepovic et al. (2016) study along with the occurrence of several *vkorc1* genes possessing two mutations such as Leu128Ser/Tyr139Cys and Ala21Thr/Tyr139Cys. The Scepovic et al. (2016) study also identified female mice as being more resistant to bromadiolone than their male counterparts. The study of Goulois et al. (2017b) investigated *vkorc1* sequences in populations of French *M. m. domesticus* and identified several resistance mutations (Ala26Ser, Glu37Gly, Arg58Gly, Trp59Gly, Leu124Met, Leu124Gln and Leu128Ser) associated with the selective pressures of using AVK rodenticides. Several of the SNPs associated with the *vkorc1*^{spr} gene (Arg12Trp, Ala26Ser, Ala48Thr and Arg61Leu) were shown to confer very limited resistance individually against FGAR and SGAR compounds (Goulois et al., 2017[b]), however, their combined effect appears to provide greater levels of resistance. Certain double mutations (i.e. the occurrence of two mutations in a single *vkorc1* sequence) were also highly associated with AVK resistance such as Ala26Ser/Leu128Ser, Trp59Gly/Leu124Met, Trp59Gly/Leu128Ser and Leu128Ser/Tyr139Cys. The Goulois et al. (2017b) study identified several individual mutations (such as Ala26Ser, Glu37Gly, Arg58Gly, Leu124Met and Leu124Gln) as only conferring a moderate level of resistance to FGARs (chlorophacinone and coumatetralyl) and limited resistance to SGARs (brodifacoum, bromadiolone, difenacoum and difethialone). In contrast to this, the mutations Leu128Ser and Tyr139Cys were found to confer significant resistance to FGARs and greater resistance to SGARs. However, it was the *vkorc1* genes containing two SNP mutations (Ala26Ser/Leu128Ser, Trp59Gly/Leu124Met, Trp59Gly/Leu128Ser and Leu128Ser/Tyr139Cys) which were found to possess the greatest broad-spectrum resistance to SGARs.

Whether a mouse is heterozygous or homozygous for *vkorc1* SNP mutations also appears to greatly affect the level of AVK resistance. Research conducted by Baxter (2019) compared AVK-tolerance to both FGARs and SGARs in *M. musculus* strains which were either homozygous or heterozygous for the Tyr139Cys resistance SNP, the findings indicated that homozygous strains displayed a much greater level of SGAR tolerance than heterozygous strains. Both the Tyr139Cys homozygous and heterozygous strains were found to display a practical level of tolerance to the SGAR bromadiolone, however the degree of resistance to bromadiolone was much greater in the homozygous strain. The homozygous strain also displayed a greater degree of tolerance to the SGARs brodifacoum, difenacoum, difethialone and flocoumafen than the heterozygous strain, indicating increased resilience. A survey of *M. m. domesticus* in Ireland revealed that 84% (42 of 50 samples) possessed either or both of the *vkorc1* mutations Leu128Ser or Tyr139Cys (Mooney et al., 2018). The survey of Irish house mice found that 14% of mice were homozygous for the Tyr139Cys mutation while 14% of the population were also homozygous for the Leu128Ser mutation. This revealed a potentially high level of resistance in the Irish wild *M. musculus* population. However, no evidence of the four mutations associated with the introgressed *vkorc1*^{spr} was detected in the Irish population. The Jones et al. (2019) report identified a high level (60%) of homozygosity of Leu128Ser and Tyr139Cys *vkorc1* resistance mutations in UK *M. musculus* populations. Jones et al. (2019) also reported a resistance prevalence of 93.2% in UK house mice populations. Blazic et al. (2019) tested the efficacy of brodifacoum for controlling bromadiolone-resistant *M. musculus* possessing *vkorc1* genes with either the Tyr139Cys mutation or the double mutation Leu128Ser/Tyr139Cys. The study confirmed the resistance conferred to bromadiolone by the SNP mutations and demonstrated that that brodifacoum (50 ppm) was still capable of controlling $\geq$90% of both male and female *M. musculus* in 2-day feeding trials.

Overall, it appears that there are several mechanisms associated with AVK resistance in *M. musculus* which are certainly influenced by gender, with females appearing more resistant to both FGARs and SGARs than their male counterparts. Additionally, there are specific single mutations to the *vkorc1* gene, such as Leu128Ser and Tyr139Cys, which confer resistance to FGARs and to a limited extent SGARs. The four *vkorc1* gene mutations associated with the introgressed *vkorc1*^{spr} gene appear to confer significant FGAR resistance and limited SGAR resistance in *M. musculus* populations. However, the presence of certain double mutations of the *vkorc1* gene such as Ala26Ser/Leu128Ser, Trp59Gly/Leu124Met, Trp59Gly/Leu128Ser and Leu128Ser/Tyr139Cys appear to confer significant resistance to SGARs. Interestingly, there may be other currently unidentified, possibly pharmacokinetic, resistance mechanism which may be separate to the *vkorc1* gene as has been hinted in the studies of Misenheimer et al. (1994) and Endepols et al. (2013).

7. Conclusion

Currently, it can be seen that there are many common AVK resistance traits shared among these three rodent species. The influence of gender, where females are inherently more tolerant of AVKs than males, appears to have been clearly established in *R. norvegicus* and *M. musculus*, while there are indications of a similar role in *R. rattus* (Garg and Singla, 2014[a]; Lefebvre et al., 2016; Scepovic et al.,2016). The gender-linked resistance phenomenon appears to be pharmacokinetic in nature, with the evidence pointing towards greater cytochrome activity and potentially longer half-lives of blood clotting factors in females than in males. However, the exact mechanism has not as yet been conclusively established. Additionally, there also appears to be a non-gender related pharmacokinetic mechanism of AVK resistance present in

each of the three species. The pharmacokinetic resistance mechanism is perhaps best described for *R. rattus,* where certain cytochrome sub-families such as CYP3A appear to be highly expressed in resistant individuals (Takeda et al., 2016). Pharmacokinetic mechanisms have also been indicated in playing a role in *R. norvegicus* resistance though the exact mechanisms (such as the CYP 450 subfamilies) remain poorly elucidated (Markussen et al., 2008). The *M. musculus* pharmacokinetic resistance mechanism also remains poorly characterised but has frequently been suspected in studies where no resistance associated *vkorc1* mutations have been observed in resistant populations (Endepols et al., 2013). Taking everything into account it seems evident that a pharmacokinetic mechanism of resistance mediated via cytochromes P-450 sub-families must play some role in AVK resistance in each of the three species. However, much more research is required on this topic in order to have a clearer understanding of which AVK pharmacokinetic mechanisms confer resistance and to what degree.

The role of SNPs altering the amino acid sequence of the *vkorc1* gene has been established in conferring some level of resistance to AVKs in the three species, although less so in *R. rattus* than *R. norvegicus* and *M. musculus*. Certain amino acid codons in exon three of the VKOR protein such as Leu120, Leu128 and Tyr139, appear to be more influential than mutations in other exons, particularly so in *R. norvegicus* and *M. musculus* (Goulois et al., 2017[b]; Hodroge et al., 2011). However, many of these mutations have yet to be shown to have developed in any of the *R. rattus* populations characterised to date. Research into *R. norvegicus* and *M. musculus* has demonstrated that individuals which are homozygous for certain mutations appear to be more resistant than heterozygous individuals (Baxter, 2019). Additionally, individuals possessing two or more different *vkorc1* mutations also appear to be more resistant to AVK compounds, although this still requires further characterisation (Goulois et al., 2017[b]). However, many resistance-conferring *vkorc1* mutations appear to come with an associated biological cost which results in impaired vitamin K cycling (Debaux et al., 2019). This may be why multiple mutations are still relatively rare in surveys of resistant rodent species. However, the introgressed *vkorc1*^{spr} allele in *M. musculus* which is highly associated with AVK resistance can contain up to four distinct mutations, albeit in exon 1 (Goulois et al., 2017a). None of the four *vkorc1*^{spr} mutations when studied individually appear to highly impair VKOR functioning or confer a high degree of resistance but taken altogether the cumulative effect appears to be highly potent (Goulois et al., 2017[b]). While *vkorc1*-mediated AVK resistance can be highly potent, many studies have shown that resistance found in certain wild rodent populations cannot be explained by *vkorc1* mutations alone and appear to have additional pharmacokinetic components (Lasseur et al., 2007).

When it comes to monitoring rodent populations for potential AVK resistance cognisance must be taken when selecting the methods by which resistance is determined. While the *vkorc1* gene provides an easily characterizable option for surveying rodent populations for potential resistance, it does not capture the full range of possible resistance mechanisms that rodents may possess. However, to date it remains the best-characterised mechanism of resistance and is a relatively cheap and efficient to establish. Yet for certain rodent species, particularly *R. rattus*, the *vkorc1* gene may not be useful in assessing the potential presence of resistance. This highlights the difficulty faced when trying to develop comprehensive resistance monitoring plans. In order to develop better resistance monitoring programmes a deeper knowledge of the currently-identified resistance mechanism is still necessary for most pest rodent species. When resistance in wild rodent populations is identified it is important to tackle these situations rapidly and effectively. Resistance management can be executed in with two principle activities (1) promoting selection in favour of anticoagulant-resistant rodents and (2) ensuring selection against resistant rodents (Greaves, 1995). This entails ceasing the use of AVKs to which rodents have become resistant and adopting alternative approaches to eliminate AVK-resistant rodents selectively and progressively.

Declaration of competing interest

The authors declare that they have no conflicts of interest in preparing this manuscript.

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Appendix A. Supplementary data

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