Chapter 8
Insecticide Resistance

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Introduction

Insecticides, including contact chemicals and fumigants, are essential components of the majority of stored product protection systems. Their use enables the implementation of effective quarantine systems, ensures food security and facilitates domestic and international trade. Insecticides have many advantages. They can be integrated easily into grain handling logistics; they reliably provide the freedom from insect infestation demanded by many markets; and they are relatively inexpensive to apply. Despite their central importance, however, there are a surprisingly small number of chemicals used in the protection of stored products. Chemical residue levels are tightly regulated as stored products are usually foods. In addition, because of the often large volumes of commodity involved and convenience of application, fumigants are frequently the preferred treatments, rather than liquid insecticides. However, fumigant use requires strict workplace health and safety precautions and must comply with stringent environmental constraints. These factors, coupled with toxicological considerations, limit the range of materials available for application to grain and make them costly to develop. For these reasons, loss of any one chemical treatment will have a significant impact on pest management. Consequently, the development of resistance in stored product pests to any registered insecticide is a particularly significant problem that requires urgent solutions.

The purpose of researching resistance phenomena is ultimately to develop strategies to prevent or delay its development or to combat it once it is manifest.

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The development of resistance to insecticides is an evolutionary phenomenon and best understood from a genetics perspective. As we will see, both molecular and population genetics approaches have driven the most recent advances in our understanding of resistance, and this has been fundamental to progress in its management.

Insecticides act on genotypic variation (through mutation, changes in chromosome structure, recombination and gene flow) to select for resistant phenotypes. Thus, our first step in understanding resistance is to understand its genetic basis. We can then examine how the selection process occurs. It is important to note that control of the selecting agent is wholly in the hands of humans. The rate at which insecticide resistance occurs is a result of human intervention (i.e. application of insecticide) that interacts with a range of factors inherent to the insects and their environment. In the presence of insecticides, individuals with a particular genotype (resistant) enjoy an advantage in survival and reproduction over other genotypes. These advantages lead to an increased genetic contribution to future generations. An individual's genetic contribution to future generations is called its fitness. Any potential fitness advantage possessed by individuals with resistance genes, however, is only apparent when the insecticide is being applied against the insect population. Insecticides are used intermittently, so we need to ask the question: what happens to gene frequencies when the insecticide is no longer being applied? Furthermore, populations of insects are rarely completely isolated. They are part of a larger ecosystem that contains other populations of the same species with different resistance gene frequencies. Therefore, factors such as insect movement and mating systems may also impact on the rate of resistance selection.

In this review, we will discuss important advances in our understanding of the genetics of resistance and the process of selection for resistance genes, and how this knowledge may help us combat resistance. We also recommend that the reviews by Boyer et al. (2012), Opit et al. (2012) and Nayak et al. (2015) to the reader to gain a comprehensive perspective of insecticide resistance in insect pests of stored products.

The Genetic Basis of Resistance

Molecular Genetics

New sequencing technologies now make it affordable to sequence whole genomes and transcriptomes (all the expressed genes, or RNA sequencing) of most organisms. This has allowed the construction of multiple insect genomes that can be used as references for research. Of the insect pests of stored products, only the genome of *Tribolium castaneum* has been sequenced with a reference sequence being made available publicly in 2008 (Richards et al. 2008), while a transcriptome of *Liposcelis bostrychophila* has also been sequenced (Dou et al. 2013; Wei et al. 2013).

Considering the increasing availability and the decreasing costs of new sequencing technologies, it is expected that many more pest insect transcriptomes and genomes will be available in the near future. Access to published reference genomes available in databases, such as GenBank or EMBL, will enable a broad range of molecular investigations into the genetic basis of an insect's physiology and ecology. The genome of *T. castaneum* has been used to identify genes responsible for pyrethroid resistance (Zhu et al. 2010) and phosphine resistance (Schlipalius et al. 2012; Jagadeesan et al. 2013).

Pyrethroid Resistance

Zhu et al. (2010) characterised a strain from Australia that was highly resistant to deltamethrin (Collins 1998). They found that the resistance was most likely metabolic and that high levels of a cytochrome P450, CYP6BQ9, a detoxification gene expressed primarily in the brain and central nervous system, were responsible for the majority of the resistance.

Phosphine Resistance

Advances in molecular genetics and DNA sequencing have enabled the identification and characterisation of the underlying genetics of phosphine resistance. The major species in which phosphine resistance has been investigated are *T. castaneum* (Jagadeesan et al. 2012, 2013) and *R. dominica* (Schlipalius et al. 2002, 2008, 2012; Kaur et al. 2012), with other species such as *Sitophilus oryzae* also starting to be investigated (Daglish et al. 2014; Nguyen et al. 2015). Resistance to phosphine has been found to be highly conserved and conferred primarily by two autosomal (i.e. not sex-linked) recessive genes, *rph1* and *rph2* (*r*esistance to *ph*osphine *1* and *2*) (Schlipalius et al. 2008). The *rph1* gene confers a weak resistance (20–30x) when homozygous and is thought to have arisen first (Schlipalius et al. 2008). The *rph2* gene also confers weak resistance when homozygous (12–20x), but acts synergistically with *rph1* to give rise to phenotypes that are strongly resistant (>250x). This two-gene requirement for strong resistance has been shown to be the case in three species investigated to date, *R. dominica* (Schlipalius et al. 2002, 2008), *T. castaneum* (Jagadeesan et al. 2012, 2013) and *S. oryzae* (Nguyen et al. 2015).

The *rph2* gene in both *R. dominica* and *T. castaneum* codes for the dihydrolipoamide dehydrogenase (DLD) gene (Schlipalius et al. 2012), which is a subunit of major metabolic enzyme complexes involved in energy metabolism, such as the TCA cycle and amino acid metabolism. These complexes are mostly mitochondrial and include pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase, branched-chain amino acid dehydrogenase and the glycine cleavage system.

In insects, the alleles conferring resistance appear to be clustered around the active site of the protein (Schlipalius et al. 2012), which suggests that it may be a

possible target site for phosphine. The observation that strains of insects with the *rph2* (DLD) resistance locus were also hypersensitive to arsenic in the form of arsine gas (Schlipalius et al. 2012), an effect that was previously observed in highly resistant *R. dominica* from Bangladesh (Chaudhry and Price 1991), supports this hypothesis. Arsenic has been shown to bind to the dihydrolipoamide cofactor of the enzyme complexes that contain DLD (Bergquist et al. 2009), which implies that phosphine is having a direct effect on the DLD enzyme. This insight into the mode of action of phosphine may be used in the future to find synergists with phosphine or to overcome resistance.

Molecular Markers

The sequence information of the *rph2* phosphine resistance gene has been used to develop molecular markers for known resistance alleles. DNA markers for phosphine resistance have already been deployed against populations from Australia (Kaur et al. 2013a), India (Kaur et al. 2015), the USA (Chen et al. 2015) and Turkey (Koçak et al. 2015). In the USA, Chen et al. (2015) reported that the marker for *rph2* resistance correlated well with the frequency of strong resistance detectable by bioassay.

Interestingly, although multiple alleles in DLD causing phosphine resistance were reported from strains isolated in Australia (Schlipalius et al. 2012), only one allele has been detected so far in other countries. This allele is the P45/49S allele (Kaur et al. 2015), which is a similar change in the protein shared at the same homologous position in both *T. castaneum* and *R. dominica*. Surveys reported so far from India (Kaur et al. 2013a), the USA (Chen et al. 2015) and Turkey (Koçak et al. 2015) show that high frequencies of this particular allele of *rph2* are common in grain storages. Although the sequences of DLD from each strain have not been reported from all these countries, it is highly likely that the allele has arisen independently in each case (Kaur et al. 2015).

There are major advantages associated with the use of molecular markers for resistance testing over the classical bioassay technique (Schlipalius et al. 2008). These include the fact that heterozygotes, or carriers of resistance that are phenotypically susceptible, can be accurately detected, and therefore, the potential for an insect population to develop strong resistance to phosphine can be assessed at a very early stage. Molecular testing has no requirement for live insects or a particular life stage, so eggs, larvae and pupae, live or dead can be assayed just as easily as live adults. This removes the need to maintain cultures of live insects caught in the field and removes the need for culturing facilities and the associated labour costs. The data generated from molecular tests are unambiguous and can be compared between surveys and laboratories easily without the requirement to develop local bioassays. There is also no requirement for a minimum number of insects per sample, as individuals can be tested. This is useful for when only one or two individuals may be detected during sampling.

Molecular markers for phosphine resistance genes now have application in routine monitoring, ecological research and evaluation of resistance management practices. Information on the frequency of resistance, the actual alleles present and their geographical distribution can be delivered in a relatively short amount of time, making these molecular markers highly valuable in resistance decision-making and contributing significantly to the preservation of phosphine as a routine treatment.

Factors Contributing to the Rate of Selection

Advances in Our Understanding of Fitness

Resistance is generally the result of the selection of relatively rare mutations that confer a fitness advantage to the insect possessing the resistance genes in the presence of insecticides. New mutations such as those coding for resistance, however, can be disruptive to the genome and may have detrimental pleiotropic effects. In the absence of insecticide, the resistance gene provides no advantage, and depending on the nature of the mutation, may be deleterious. That is, there may be a fitness cost associated with the resistance gene in the absence of insecticide. The actual effects may be physiological or even behavioural. Identifying fitness costs associated with resistance is important in designing resistance management strategies because the higher the fitness cost, the longer it is likely to take for resistance to spread in the population (Klior and Ghanim 2012). Differences in fitness between resistant and susceptible genotypes are assumed in the design of key resistance management tactics such as alternation of insecticides (Onstad 2008).

Pyrethroid Resistance

The nature of fitness costs has been investigated in maize weevil strains, *Sitophilus zeamais*, from Brazil, with high levels of resistance to pyrethroid insecticides. The Juiz de Fora strain had reduced and delayed emergence, reduced population growth and consumed less maize compared with two susceptible strains. That is, there appeared to be a fitness cost associated with resistance to pyrethroids in this strain. This was in contrast to the Jacarezinho strain, in which there was no apparent cost associated with resistance. This strain showed similar population growth rate and development time to susceptible strains (Fragoso et al. 2005). The dominant resistance mechanism in both strains was target site insensitivity (Guedes et al. 1995). The contrast between these two strains presented an opportunity to identify the physiological basis for fitness costs (Guedes et al. 2006), that is, to answer the question: was there an energy trade-off between insecticide resistance and other processes associated with development and reproduction? To do this, Guedes et al. (2006) measured respiration rate and fat body morphology as indicators of energy

requirements and capacity in these two strains and compared their rates of development. They found that the Jacarezinho strain (no-cost) had higher body mass and larger fat body providing higher energy reserves than Juiz de Fora, and in addition, this strain had higher respiration rate indicating better mobilisation of energy. They postulated that the extra energy available to the insect compensates for the additional energy requirements needed to support resistance mechanisms without compromising demographic performance. In contrast, the Juiz de Fora strain was smaller in size, had lower respiration rate and had lower demographic performance indicating a physiological cost associated insecticide resistance. Other adaptations shown by the Jacarezinho strain included higher amylase activity, which could contribute to more efficient breakdown of starches (Araujo et al. 2008a), and more efficient digestive enzymes (Araujo et al. 2008b).

In addition to physiological differences, a resistance genotype may also affect insect behaviour. Pyrethroid-resistant *S. zeamais* were found to better detect the presence of deltamethrin than susceptible insects, being less likely to feed on deltamethrin sprayed grain when given a choice (Guedes et al. 2009a). In no-choice experiments, one pyrethroid-resistant strain continued to feed on treated grain at higher concentrations but another ceased feeding. Guedes et al. (2009a) suggested that the continued feeding in the former strain may have been to compensate for energy expended protecting against the insecticide. In several other studies, no correlation between physiological resistance to insecticides and other behavioural parameters, such as flight initiation and walking, could be found in *S. zeamais* (Periera et al. 2009; Guedes et al. 2009b; Braga et al. 2011; Corrêa et al. 2014), although it appears that higher walking activity and flight initiation may be associated with increased insect weight in this species (Guedes et al. 2009b).

Phosphine Resistance

An indication of possible fitness deficit associated with phosphine resistance was provided by Pimentel et al. (2007) who reported correlations between respiration rate, rate of reproduction and phosphine resistance ratios at the LC_{50} in field-collected samples of *T. castaneum*, *R. dominica* and *Oryzaephilus surinamensis*. For all species, respiration rate decreased as resistance ratio increased, while instantaneous rate of population growth decreased as resistance factor increased. More definitive evidence was provided by Kaur et al. (2012) who identified increased delays in development of immature stages after fumigation with phosphine in the strong phosphine resistance strain of *R. dominica*. The delay was inherited in a similar manner to the toxicity response and appeared to be a pleiotropic effect of phosphine resistance.

A possible link between movement and phosphine resistance in *R. dominica* was proposed by Pimentel et al. (2012) who found that walking activity was significantly reduced after exposure to phosphine in one phosphine-resistant strain compared with a susceptible strain but not in another. In contrast, Kaur et al. (2013a, b) found no evidence of any link between phosphine resistance and

duration of walking or flight initiation in genetically characterised strong resistant, weak resistant and susceptible strains.

A simple approach to detecting fitness differences associated with resistance genes is to establish a 'population cage'. Typically in these experiments, homozygous resistant and susceptible strains are crossed and then bred through a series of generations without exposure to the insecticide. A representative sample of insects is tested at intervals to detect any change in the frequency of resistant and susceptible genotypes or phenotypes. Using this method revealed no evidence of fitness deficits in *R. dominica* over 20 generations (Schlipalius et al. 2002) or *S. oryzae* (Daglish et al. 2014) or *T. castaneum* (Daglish et al. 2015) over seven generations. Jagadeesan et al. (2012) crossed homozygous weak resistant and strong resistant strains of *T. castaneum* with a susceptible strain. They found no fitness deficit associated with *rph1* but there was an indication of a fitness cost linked with *rph2*. This was confirmed in a follow-up study (Jagadeesan et al. 2013) where the authors observed a significant decrease in the frequency of *rph2* homozygous resistant genotype in *T. castaneum* over 18 generations with a corresponding increase in heterozygote and susceptible genotypes indicating a selective fitness disadvantage for homozygotes at the *rph2* locus. In contrast, the authors observed a significant increase in the frequency of the homozygous *rph1*, suggesting a fitness advantage of weakly resistant homozygotes compared to susceptible genotypes.

What Have We Learned About Fitness?

Fitness deficits associated with resistance genes can, theoretically, impact on the rate of selection of resistance in insect populations, and are therefore important to consider when developing resistance management strategies. Recent research demonstrates that there may be fitness differences between resistant and susceptible insects but the evidence is generally inadequate. There appears to be a deficit associated with phosphine resistance in some species, perhaps an allele of *rph2*, expressed as delayed immature development (Pimentel et al. 2007; Jagadeesan et al. 2012; Kaur et al. 2013a, b). It is yet to be determined, however, if this effect would be significant in the practical management of resistance to phosphine. Evidence from population cage experiments suggests that it may not (Schlipalius et al. 2002; Daglish et al. 2014). Severe fitness deficit has also been associated with a pyrethroid-resistant strain of *S. zeamais* (Guedes et al. 2006); however, it appears to be quite limited in frequency. It should be noted evolution is not static and deleterious effects associated with resistance may be reduced by the selection of modifier genes that may interact with the resistance genes producing a positive epistasis. Thus, fitness disadvantage associated with resistance alleles, perhaps obvious in the initial stages of selection, may become undetectable over generations as selection for an optimum phenotype occurs.

The methods used to detect and characterise fitness traits in insects are critical. Many factors can affect life history and behavioural parameters and the expression

of these characteristics can vary significantly from strain to strain, independent of resistance status. This is particularly apparent when comparing long maintained laboratory reference strains with recently derived field strains which have been under quite different selection pressures. These pressures can result in significant differences in life history traits and other factors such as disease contamination. The best way to minimise genetic and other differences between strains is to compare strains that share a similar genetic background except for the character of interest. These can be created by either isolating two or more lines from one field strain, preferably one line with the resistance gene(s) and one without, usually through single pair mating and selection, or by creating isogenic or introgressed lines. The latter are created through repeated back-crossing of a resistant parent into a susceptible strain with selection. Many studies suffer from poorly defined genotyping and from the possibility of unknown background strain effects influencing experimental results. In addition, laboratory-based studies may not account for fitness aspects that are important under field conditions, such as disease susceptibility or temperature fluctuations.

Population Structure and Gene Flow

The development of molecular resistance gene markers (Schlipalius et al. 2012; Jagadeesan et al. 2013; Kaur et al. 2013a) is facilitating much more accurate assessments of resistance gene frequencies within populations of insect pests of stored products (Jagadeesan et al. 2013; Kaur et al. 2013a; Chen et al. 2015; Koçak et al. 2015); however, these markers have not yet been used to investigate resistance gene flow. Nevertheless, gene flow may be inferred from studies of population dispersal supported by neutral DNA marker studies. As well as being carried along transportation routes, the major pest species *T. castaneum*, *C. ferrugineus* and *R. dominica* are active flyers and are known to readily disperse relatively long distances (Mahroof et al. 2010; Semeao et al. 2010; Ridley et al. 2011; Daglish et al. 2014). Comparisons of individual insects across landscapes using neutral DNA markers indicate gene flow over very broad areas (Drury et al. 2009; Ridley et al. 2011) supporting the need for implementation of management strategies on a regional scale. Biological factors may also contribute significantly to rates of resistance selection. For example, one study revealed that 97% *T. castaneum* and *R. dominica* emigrating from grain storages had mated, and most with more than one male (Walter et al. 2014), increasing the likelihood that their progeny carries resistance genes. Furthermore, experimental evidence (Kaur et al. 2013a, b) indicates that phosphine-resistant insects can disperse as actively as susceptible insects. Sublethal effects are also likely to be important as it has been demonstrated that prior exposure to relatively low concentrations of phosphine reduces fecundity in phosphine-resistant *T. castaneum* (Ridley et al. 2012a) and *R. dominica* (Ridley et al. 2012b). It is now clear that biological and ecological factors and their interactions can impact significantly on resistant gene flow and on insecticide

selection in populations of insect pests of stored products. The results of these studies are central to our understanding of the selection and distribution of resistance and, therefore, provide valuable information for the development of resistance management strategies.

Managing Resistance—Modelling the System

Laboratory studies can reveal important information about the factors that drive resistance development such as its inheritance, dominance and any fitness aspects associated with it, and field studies can provide insights into the dispersal of insects in the landscape and gene flow. However, because of the very large numbers of insects involved, the considerable number of potential variables, including those imposed by humans, and the long time frames required to include realistic numbers of insect generations, controlled experiments designed to test resistance management strategies are necessarily quite limited in scope. Simulation modelling, however, offers a method of evaluating a range of tactics and strategies under various scenarios that can produce credible and useful results if based on realistic data.

Rather than taking a theoretical approach based on general assumptions as was common in the past, recent model development has been based on information about real resistance phenomena, in particular, resistance to phosphine in the lesser grain borer, *R. dominica*. Resistance to phosphine in this species is controlled by two major genes (Collins et al. 2002; Schlipalius et al. 2002) and represents a serious resistance threat (Collins et al. 2000). Both Lilford et al. (2009) and Shi et al. (2012a) independently demonstrated that models based on the real-life two-gene situation in this species, rather than the single-locus resistance assumption used in previous modelling, much more accurately matched theoretical predictions of genotypes which subsequently affected model predictions, thus supporting the need for accurate experimental analysis of resistance genetics. Both models also used published phosphine mortality responses for *R. dominica* (Collins et al. 2002; Daglish 2004; Collins et al. 2005) to estimate resistance factors for various genotypes and to model survival rates (Lilford et al. 2009; Shi et al. 2011).

The Lilford two-locus model (Lilford et al. 2009) consisted of nine subpopulations, corresponding to nine genotypes, modelled by a system of nonlinear ordinary differential equations. Although the model was relatively simple, being based on responses of a single life stage and homogeneous fumigant concentrations, temperature, etc., simulations of fumigations matched field observations and expected gene frequencies. The model was expanded to include the response of all life stages (Thorne et al. 2010) providing insights into gene frequencies and population recovery between fumigations and strategic timing of treatments.

A different approach to modelling phosphine resistance in *R. dominica* was taken by Shi and collaborators who developed a stochastic, individual-based model (Shi et al. 2011, 2012a, b, 2013). These models represent the fact that insect populations consist of individual beetles, each of a particular genotype and life stage (Shi

et al. 2012a). The advantage of this approach over simpler population-based models is that it allows more aspects of individual variability and biological reality to be included. The Shi model also used a daily time step, which can capture real conditions in more detail and obtain more precise results than a weekly time step. In the real world, conditions are more likely to change day to day than week to week (Shi et al. 2012b). Using this model, Shi et al. investigated management tactics relating to both single and successive fumigations. They concluded that fumigation for a longer period and at lower concentration is more effective than a shorter fumigation at a higher concentration. This is because under the former, eggs and pupae have time to develop to less tolerant stages (this assumes no delay in development under fumigation, or effect on fecundity). In addition, in a two-gene system where dilution of resistance genes through immigration has a greater effect, extending fumigation times will have a significant impact on delaying the development of resistance. Shi et al. (2012b) also examined the impact of initial resistance gene frequency and initial number of insects on fumigation success. They found that the rate of insect survival increases proportionally with initial genotype frequency and that if the original frequency of homozygous resistant insects is increased n times, then the fumigation needs to be extended n days to achieve the same level of control. Likewise, to achieve a similar level of control of a population that is n times larger than another population, the fumigation time needs to be increased by approx. n days. This demonstrates that increasing the duration of fumigation is an efficient way to increase efficacy, a conclusion matched by experimental evidence (Collins et al. 2005).

The authors then examined the impact of two other important factors and their interaction: fumigation dosage consistency within a storage and the effect of insect immigration into the storage, on resistance frequencies and insect numbers. This analysis was over 732 days and included a series of six fumigations within that time. The consistency of dosage, that is, distribution of fumigant, was the key factor in avoiding evolution of resistance and suppressing population increase. When consistency was high (even distribution of fumigant) there was no increase in the frequency of resistance or population numbers, regardless of immigration rate. When immigration is excluded, selection of resistance occurs faster in storages with moderate dosage inconsistency than in storages with low dosage inconsistency. In moderate dosage inconsistency, overall numbers increase but this increase is slow. In storages with low dosage consistency, that is, a leaky storage or one within which the gas is very unevenly distributed, population numbers increase because more insects survive, and resistance frequencies increase with every fumigation because of selection. Immigration of susceptible insects has a dilution effect but not enough to counteract the increase in resistance frequencies, even at the highest immigration rates (100 insects/day). The practical significance of this analysis is that storages should be well sealed before fumigation and active mechanisms used to distribute phosphine evenly to achieve high dosage consistency. In addition, insect movement into storages should be reduced as much as possible.

Ideally, models of resistance to insecticides should allow simulation and analysis of real-life storage situations in which variables can be changed to create a range of scenarios to test resistance management assumptions and tactics. The models

described here incorporated genotype response data and life table information. The predicted outcomes matched the experimental evidence and industry experience demonstrating their validity and potential to be used to evaluate resistance management tactics. The models also highlighted key gaps in our knowledge thus helping to guide the direction of research. Nevertheless, these models are relatively simple and to adequately simulate fumigations of stored grain many more factors, such as physical characteristics of the fumigant (e.g. leakage, distribution and sorption) and biological influences, such as the effects of temperature and sublethal exposure on insect biology, need to be included, as well as the response of insects to various management interventions.

Conclusions

The most important recent advances in our knowledge of insecticide resistance have been in our understanding of genetics of resistance, particularly to phosphine, and the development of advanced molecular tools for diagnosing resistance. This has also led to the discovery of the underlying mechanisms involved in phosphine resistance and new knowledge of the mode of action of phosphine, which was not previously well understood. These insights will provide a basis for the development of new chemical tactics. Our understanding of selection of resistance genes and gene flow processes in populations, however, is at an early stage. Some progress has been made in our knowledge of relative fitness in particular; however, the importance of this issue is not resolved. The research on biological factors that may influence selection rates and gene flow emphasise the importance of these studies but there are still many gaps in our knowledge. Simulation modelling offers a method of integrating our knowledge of insect biology and ecology, genetic processes and insect responses to insecticides with the action of the insecticide on the commodity and within the storage. Models will become more powerful and predictive as more information is added but their usefulness relies on the quality of experimental data available, and there are still many gaps. However, models have the potential to be very valuable tools for understanding resistance development, testing resistance management tactics and devising resistance management strategies.

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