UNIVERSITY OF THE WITWATERSRAND

# THE BIOLOGICAL ROLE OF AKIRIN IN *ANOPHELES ARABIENSIS*

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# DECLARATION

I, Blaženka Danica Letinić, declare that this Thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

.....(Signature of candidate)

17th day of November in 2020

# DEDICATION

In Loving Memory of My Father

**SVETKO LETINIĆ**

(1 November 1937 - 18 February 2017)

# PUBLICATIONS AND PRESENTATIONS

## PUBLICATIONS

**Letinić, B.D., Contreras, M., Dahan-Moss, Y., Linnekugel, I., de la Fuente, J. and Koekemoer, L.L.**, 2020. Characterising the effect of three different recombinant Akirin vaccines on *Anopheles arabiensis. Parasites & Vectors*, (submitted and under review).

**Letinić, B.D., Dahan-Moss, Y. and Koekemoer, L.L.**, 2020. Characterising the effect of *akirin* knockdown on *Anopheles arabiensis* (Diptera: Culicidae) reproduction and survival, using RNA-mediated interference. *PloS One, 15*(2), p.e0228576.

**Letinić, B.D., Kemp, A., Christian, R.N. and Koekemoer, L.L.**, 2018. Inoculation protocol for the African malaria vector, *Anopheles arabiensis,* by means of nano-injection. *African Entomology*, *26*(2), pp.422-428.

## ORAL PRESENTATIONS

**Letinić, B.D., Contreras, M., Dahan-Moss, Y., Linnekugel, I., de la Fuente, J. and Koekemoer, L.L.**, 2020. Characterising the effect of three different recombinant Akirin vaccines on *Anopheles arabiensis.* Faculty Research Day, University of Witwatersrand, Johannesburg, Gauteng, South Africa, 15 October.

**Letinić, B. D., Dahan-Moss Y. L., Koekemoer, L. L.**, 2019. Akirin immunisation for the control of *Anopheles arabiensis* infestations. 9th Molecular Biosciences Research Thrust, University of Witwatersrand, Johannesburg, Gauteng, South Africa, 28 November.

**Letinić, B. D., Dahan-Moss Y. L., Koekemoer, L. L.**, 2019. *Akirin* knockdown: a potential tool for *Anopheles arabiensis* vector control. 21st Entomology Society of Southern Africa Congress, Durban, KwaZulu-Natal, South Africa, 8 – 11 July.

**POSTER PRESENTATIONS**

**Letinić, B. D., Christian, R. N., Koekemoer, L. L.**, 2018. The biological effects of *akirin* knockdown on the African malaria vector, *Anopheles arabiensis.* Faculty Research Day, University of Witwatersrand, Johannesburg, Gauteng, South Africa, 6 September.

**Letinić, B. D., Christian, R. N., Koekemoer, L. L.**, 2018. Using *akirin* knockdown as an additional tool for *Anopheles arabiensis* vector control. 4th Malaria Research Conference, National Institute for Communicable Diseases, Johannesburg, Gauteng, South Africa, 30 July – 1 August.

**Letinić, B. D., Christian, R. N., Koekemoer, L. L.**, 2018. The biological role of Akirin, in *Anopheles arabiensis,* at both a phenotypic and a transcriptional level, using RNA interference. SASBMB-FASBMB, North-West University, Potchefstroom, North-West, South Africa, 8 – 11 July.

**Letinić, B. D., Christian, R. N., Koekemoer, L. L**., 2017. Optimised nano-injection inoculation protocol for the African malaria vector, *Anopheles arabiensis*. 3rd Malaria Research Conference, National Institute for Communicable Diseases, Johannesburg, Gauteng, South Africa, 7 – 9 November.

**Letinić, B. D., Christian, R. N., Koekemoer, L. L.**, 2017. The biological role of Akirin in *Anopheles arabiensis,* at both a phenotypic and a transcriptional level, using CRISPR interference. 3rd International Congress on Parasites of Wildlife, Kruger National Park, Skukuza, Mpumalanga, South Africa, 24 – 27 September.

**Letinić, B. D., Christian, R. N., Koekemoer, L. L.**, 2016. Akirin genes as a novel control of the African malaria vector *Anopheles arabiensis*. 6th Molecular Biosciences Research Thrust, University of Witwatersrand, Johannesburg, South Africa, 8 December.

# ABSTRACT

*Anopheles arabiensis* is an opportunistic malaria vector that rests and feeds outdoors, circumventing current vector control methods. Furthermore, this vector will readily feed on animal as well as human hosts. Agricultural animals are regularly vaccinated with recombinant proteins, for the control of multiple endo- and ectoparasitic infestations. Targeting *An. arabiensis,* while feeding on animals, can provide an additional intervention to the current vector control activities.

Previously, the use of a subolesin-vaccine showed a mark reduction in tick survival and reproductive fitness. Targeting Akirin (Subolesin ortholog) in *An. arabiensis,* when performing CRISPR mediated interference or RNA mediated interference, reduced both vector survival and reproductive capacities. This gene regulation function made Akirin a potential antigen for vaccine development against *An. arabiensis,* which was further investigated.

The efficacy of three recombinant Akirin vaccines was evaluated, to determine if Akirin could represent a novel target for the control of *An. arabiensis*. Immunisation trials were conducted based on the concept that female *An. arabiensis*, feeding on vaccinated hosts (balb/c mice), would ingest antibodies specific to the target antigen, affecting its function within the vector. All three antigens evaluated, namely recombinant Akirin from *An. arabiensis*, recombinant Akirin from *Aedes albopictus* and recombinant Q38 (Akirin/Subolesin chimera), successfully reduced *An. arabiensis* survivorship and reproductive capacities, with a vaccine efficacy of 68 to 73%.

In conclusion, these results were the first to characterise the biological role of Akirin in *An. arabiensis* survival and reproduction, by performing CRISPRi and RNAi, using an optimised nano-injection protocol. Furthermore, these results were the first to show that blood hosts vaccinated with recombinant Akirin vaccines could aid in the suppression of *An. arabiensis* vector populations, through the reduction of vector survivorship and reproductive capacities. Therefore, these results provide a step towards the development of a novel target for *An. arabiensis* vector control.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AEC | Animal Ethics Committee |
| Ago2 | Argonaut-2 |
| Akirin$^{albopictus}$ | Recombinant Akirin from *Ae. albopictus* |
| Akirin$^{arabiensis}$ | Recombinant Akirin from *An. arabiensis* |
| AMP | Antimicrobial peptide |
| AMV | Avian Myeloblastosis Virus |
| ANK | Ankyrin-repeat rich domain |
| ANOVA | Analysis of variance |
| β2m | Beta-2 microglobulin |
| BLAST | Basic local alignment search tool |
| BSA | Bovine serum albumin |
| Cas | CRISPR-associated |
| Cascade | CRISPR-associated complex for antiviral defence |
| cDNA | Complementary DNA |
| CMR | Cas RAMP proteins |
| Cq | Quantification cycle |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| CRISPRi | CRISPR interference |
| DAP-PGNs | Meso-diaminopimelic acid type peptidoglycans |
| dCas9 | Nuclease-deficient Cas9 |
| DDT | Dichlorodiphenyltrichloroethane |
| DF | Degrees of freedom |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate's |
| DREDD | Death-related ced-3/nedd2-like protein |
| dsDNA | Double-stranded DNA |
| dsRNA | Double-stranded RNA |
| ELISA | Enzyme-linked immunosorbent assay |
| E-value | Efficiency-value |
| FADD | Fas-associated protein with death domain |
| FPLC | Fast protein liquid chromatography |

| | |
|---|---|
| HDR | Homology-directed repair |
| HNH | An endonuclease domain named for characteristic histidine and asparagine residues. |
| HRP | Horseradish peroxidase |
| IAP1 | Inhibitor of apoptosis protein 1 |
| IAP2 | Inhibitor of apoptosis protein 2 |
| IKK | Inhibitor of κB kinase |
| IMD | Immune deficiency |
| IPTG | Isopropyl-β-D-1-thiogalactopyranoside |
| IRS | Indoor residual spraying |
| ITNs | Insecticide-treated nets |
| LB | Lysogeny broth |
| MBN | A laboratory strain of *An. arabiensis* mosquitoes originally collected using a man baited net |
| miRNAs | MicroRNAs |
| mRNA | Messenger RNA |
| mRNA | Messenger RNA |
| NCBI | National centre for biotechnology information |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B-cells |
| NHEJ | Non-homologous end-joining |
| NHLS | National Health Laboratory Service |
| OD | Optical density |
| NICD | National Institute for Communicable Diseases |
| PAM | Protospacer-adjacent motif |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PGRP | Peptidoglycan recognition protein |
| piRNAs | Piwi-interacting RNAs |
| pre-crRNAs | Precursor CRISPR RNAs |
| Q38 | Subolesin/Akirin chimera |
| RdRP | RNA-dependent RNA polymerase |
| RHD | Rel homology domain |
| RIN | RNA integrity number |

| | |
|---|---|
| RISC | RNA-induced silencing complex |
| Rpm | Revolutions per minute |
| RNA | Ribonucleic acid |
| RNAi | RNA mediated interference |
| RNase III | Ribonuclease III |
| RT-qPCR | Reverse-transcription quantitative PCR |
| RuvC1 | An endonuclease domain named for an *E. coli* protein involved in DNA repair |
| S.E. | Standard error |
| SAVC | South African Veterinary Council |
| SAVP | South African Vaccine Producers |
| SDS-PAGE | Sodium-dodecyl-sulphate polyacrylamide-gel-electrophoresis |
| sgRNA | Single-guide RNA |
| SID-1 | Systemic RNA interference deficiency-1 |
| siRNAs | Short interfering RNAs |
| SOC | Super optimal broth with catabolite repression |
| ssDNA | Single-stranded DNA |
| TAB2 | TAK1-binding protein1 |
| TAE | Tris/Acetate/EDTA |
| TAK1 | TGF-β activated kinase 1 |
| *Taq* | *Thermus aquaticus* |
| TBE | Tris/Borate/EDTA |
| TBS | Tris-buffered saline |
| TBS-T | TBS-Tween 20 |
| *Tfl* | *Thermus flavus* |
| tracrRNA | Trans-activating CRISPR RNA |
| TSS | Transcriptional start site |
| Txn | Transcription |
| Ub | Polyubiquitinated |
| VCRL | Vector Control Reference Laboratory |
| WHO | World Health Organization |

# CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

## 1.1 Malaria

The World Health Organization (WHO) stated that in 2018 there were approximately 228 million cases of malaria worldwide, of which 93% occurred within the African region. Like many other febrile illnesses, malaria characteristically presents as a fever accompanied by chills, sweats, headaches, and vomiting. If left untreated, symptomatic malaria infections may become more severe, resulting in anaemia, hypoglycaemia, metabolic acidosis, multiple organ failure, convulsions, loss of consciousness, or death (World Health Organization, 2019). The presence of a fever is the premise for suspicion of malaria in patients residing in regions where malaria is prominent, and is also a basis for prompt diagnostic testing. Patients with suspected malaria should undergo blood smear microscopy (Metcalf, 1945) or rapid diagnostic testing (Beadle *et al.,* 1994) to corroborate the presence or absence of *Plasmodium* parasites within the bloodstream (Warhurst and Williams, 1996; World Health Organization, 2019).

*Plasmodium* enters the human bloodstream by the bite of an infected female *Anopheles* mosquito, in the form of sporozoites (Manson, 1898). The sporozoites migrate to the liver, where they infect the hepatocytes. After undergoing several nuclear divisions, schizonts containing hepatic merozoites are formed (Laveran, 1880). The hepatic merozoites are released into the bloodstream, where they consequently invade nearby erythrocytes (Laveran, 1880; Councilman, 1887). In the erythrocytes, several daughter merozoites are produced. Upon rupturing the host erythrocytes, the daughter merozoites are released into the bloodstream, allowing them to further invade subsequent erythrocytes (Laveran, 1880; Ross, 1896; Manson, 1898; World Health Organization, 2019). This asexual cycle causes the symptoms associated with malaria infection.

*Plasmodium falciparum* infections display symptoms 10-21 days after being bitten by an infected mosquito, while *P. malariae, P. ovale, and P. vivax* infections may take up to 12 months to manifest clinically (Bass and Johns, 1912; Blacklock, 1921; Church *et al*., 1997; Faye *et al*., 2002; Koltas *et al*., 2007). *Plasmodium falciparum* is the most prevalent malaria parasite on the African continent, accounting for 99.7% of the overall malaria cases in the

WHO African Region, and 100% of malaria cases that were reported in South Africa for 2018 (World Health Organization, 2019).

Uncomplicated malaria infections are treated using artemisinin-based combination therapy. Artemisinin is used to reduce the abundance of *Plasmodium* within the bloodstream during the first three days of treatment (Klayman, 1985; Meshnick *et al*., 1996; Price *et al*., 1996), while a partner drug is used to clear any remaining parasites (World Health Organization, 2019). There are five different artemisinin-based combination therapies recommended by the WHO: artesunate-amodiaquine, artemether-lumefantrine, artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine, and dihydroartemisinin-piperaquine (World Health Organization, 2020[a]). Patients who display symptoms of severe malaria require immediate hospital admission, where artesunate can be administered intravenously for 24-hours, followed by a course of artemether-lumefantrine oral treatment (Shah and Chittora, 1986; World Health Organization, 2019). This reactive solution has subsequently contributed to the success of malaria treatment globally.

However, although malaria is both avoidable and curable, an estimated 405,000 people succumb to malaria annually, with children below the age of five accounting for 67% of all malaria deaths globally (World Health Organization, 2019). Between 2015 and 2018, household surveys were conducted in 20 sub-Saharan Africa countries, where it was shown that only 42% of children with febrile were taken to a trained medical practitioner for examination (World Health Organization, 2019). This was due to a lack of awareness of malaria symptoms among caregivers, and was also due to poor access to health care facilities.

Given the anticipated escalation of the world's population, more people will be residing in countries where malaria is a risk, putting additional strain on health care systems and financial recourses (World Health Organization, 2019). Therefore, proactive solutions, such as vector control, remain the primary approach in reducing malaria transmission, as the high morbidity and mortality rates of malaria are mainly attributed to the geographical distribution of the anthropophilic human malaria vectors in each region.

Africa experiences the bulk of the global malaria burden, due to the efficiency of the dominant malaria vector species in each region. *Anopheles moucheti* is a dominant vector found in the equatorial forest regions of Cameroon and Nigeria (Antonio-Nkondjio *et al*., 2008; Sinka *et*

*al.,* 2010), whereas *An. nili* is a dominant vector commonly found across West, Central, and East Africa, in the humid savanna and degraded rainforest regions (Gillies and Coetzee, 1987; Sinka *et al.,* 2010). *Anopheles funestus* is widely distributed across the African continent and often occurs in sympatry with the dominant vector species belonging to the *An. gambiae* complex (Coetzee and Fontenille, 2004; Sinka *et al.,* 2010).

The *An. gambiae* complex is comprised of nine species in total (Gillies and Coetzee, 1987; Coetzee *et al.,* 2013[a]; Coetzee, 2020). Both *An. amharicus* and *An. quadriannulatus* are zoophilic in behaviour, and are not considered vectors of human malaria (Sinka *et al.,* 2010), whereas *An. bwambae* and *An. fontenillei,* are highly restricted in their distribution and only occur in western Uganda and Gabon respectively (White, 1985; Barrón *et al*., 2019). The saltwater tolerant coastal species, *An. melas* and *An. merus,* are both minor malaria vectors and can be found along the west and east coastal regions, respectively. This includes regions along the Gambia River in West Africa (Muirhead-Thomson, 1946; Muirhead-Thomson, 1948; Gelfand, 1955; Giglioli, 1964), as well as the Tanzanian, and Mozambique coast, stretching as far south as South Africa (Gillies and Coetzee, 1987; Temu *et al*., 1998; Cuamba and Mendis, 2009). Therefore, the three dominant vector species in Africa are *An. gambiae s.s., An. coluzzii* and *An. arabiensis* (Gillies and Coetzee, 1987; Coetzee *et al.,* 2013[a]).

In South Africa, malaria transmission primarily transpires in the low altitude regions of the KwaZulu-Natal, Limpopo, Mpumalanga provinces, which border Eswatini, Mozambique, and Zimbabwe (National Department of Health, 2019). Historically, the main malaria vectors in South Africa were *An. funestus* and *An. arabiensis* (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Irish *et al*., 2020). *Anopheles funestus* is the cause of malaria outbreaks and epidemics in South Africa, while *An. arabiensis* was largely accountable for low-level seasonal transmission in the malaria-affected regions (Brooke *et al.,* 2013). Effective vector management has since eliminated *An. funestus* from South Africa, leaving *An. arabiensis* as the primary malaria vector (Mouatcho *et al.,* 2007; Dandalo *et al*., 2017).

## 1.2 Malaria Vector Control

Malaria vector control efforts are currently aimed at interventions that prevent human-vector contact (Sinka *et al.,* 2010). These interventions are primarily insecticide based. After the Second World War, the use of dichlorodiphenyltrichloroethane (DDT), an organochlorine, was upscaled for agricultural and antimalaria house spraying purposes (Pampana, 1969). This resulted in the emergence of DDT resistance amongst various vector populations, which led to the manufacturing and development of alternative insecticides such as carbamates, organophosphates, pyrethroids, and pyrroles (World Health Organization, 1997).

Currently, pyrethroids and pyrroles are the only classes of insecticide permitted by the WHO for the impregnation of long-lasting insecticide-treated bed nets (LLINs) (Oxborough *et al.*, 2015; World Health Organization, 2019). This preventative measure is designed to physically prohibit human-vector contact by forming a protective barrier when beneath it. The insecticides impregnated in the fibres of the nets either kill or repel susceptible mosquitoes, reducing the amount of anthropomorphic endophilic malaria vectors. Indoor residual spraying (IRS) is another preventative method used to impede human-vector contact. This preventative measure is executed by spraying insecticides on the walls of residences to reduce the amount of endophilic and endophagic malaria vectors. These interventions have been proven both efficient and effective where aptly implemented (World Health Organization, 2019). However, the increasing prevalence of insecticide resistance in target vector populations tend to undermine these efforts.

Insecticide resistance refers to the change in an insect's ability to withstand or overcome the toxic effects of one or more insecticides, due to specific traits or behaviours acquired by means of natural selection or genetic mutation (World Health Organization, 1997). The cumulative use of insecticides for vector control, coupled with the continued use of agricultural pesticides, has led to the selection of resistance genes in many vector populations over the past decade (Brooke *et al.,* 2013). It has been reported that 90% of the malaria vector population in the WHO African regions are resistant to at least one of the four insecticide classes, while 32% of the vector population is resistant to all four insecticide classes (World Health Organization, 2019). This could potentially result in malaria vectors not being affected when encountering a standard dose of an insecticide deployed through LLINs or IRS. These reports display the importance of resistance monitoring and of vector control management strategies.

*Anopheles funestus* had been eliminated from South Africa since the 1950s due to vector control management using DDT (Olivier, 1950). However, in the 1960s, DDT began receiving more criticism concerning its harmful effects on people and the environment, causing South Africa to shift its malaria control programmes towards the use of pyrethroids (Sharp and le Sueur, 1996; Hargreaves *et al.,* 2000). Unknown at the time, the *An. funestus* population from southern Mozambique was resistant to pyrethroids. This caused a malaria epidemic in northern KwaZulu-Natal, which gave rise to just under 65,000 malaria cases in 2000 (Coetzee *et al*., 2013[b]).

South African malaria vector control moved towards an IRS mosaic approach, where pyrethroids were used to treat cement-brick structures, and DDT was used to treat traditional mud-walled structures (Brooke *et al.,* 2013). The use of DDT once again eliminated *An. funestus* from South Africa, while pyrethroids were used to control the *An. arabiensis* population, in northern KwaZulu-Natal, as these vectors were resistant to DDT but susceptible to pyrethroids (Mouatcho *et al.,* 2007; Mouatcho *et al.,* 2009).

South Africa has since observed substantial reductions in malaria transmission, with approximately 7,000 malaria case incidences being reported annually (Brooke *et al.,* 2013). However, this rose to 22,061 cases of malaria in 2017, due to an epidemic that broke out in the Limpopo and Mpumalanga provinces (National Department of Health, 2019). There has since been a 43% reduction in malaria case incidence, as only 9,562 cases of malaria were reported in 2018 (World Health Organization, 2020[b]).

Most of the cases reported in 2018 were due to the cross-border movement of populations from high-risk areas. Imported malaria cases constitute approximately 60% of the total annual cases reported in South Africa (World Health Organization, 2020 [b]). Given the high rate of imported malaria, collaborations with neighbouring malaria-endemic countries are essential to decrease the malaria importation risk, and prevent local transmission. Regional programs have subsequently been set-up to sustain and strengthen cross-border operational committees, through harmonisation of cross-border operations, and through coordination of cross-border policies (Sharp *et al.,* 2007; Maharaj *et al.,* 2012; National Department of Health, 2019).

While these initiatives are needed to reduce the rate of imported malaria in South Africa, approximately 40% (3,798 cases) of the malaria cases reported in 2018 were due to local transmission (World Health Organization, 2020 [b]). The low-level seasonal malaria transmission experienced in South Africa is attributed to the opportunistic malaria vector *An. arabiensis* (Brooke *et al.*, 2013; Dandalo *et al*., 2017).

*Anopheles arabiensis* shows discrepancies in feeding behaviour (endophagic, exophagic) and resting behaviour (endophilic and exophilic) when compared to other *Anopheles* malaria vector species (Gillies and Coetzee, 1987). Its behavioural plasticity allows *An. arabiensis* to evade current control methods as they are unaffected by IRS (Brooke *et al.*, 2013). This problem is further compounded due to escalating insecticide resistance in the *An. arabiensis* vector population (Casimiro *et al*., 2014; Brooke *et al.*, 2015; Mzilahowa *et al*., 2016; Messenger *et al.*, 2017).

Recently, the *An. arabiensis* population in South Africa has displayed resistance to DDT, carbamates, and pyrethroids, particularly in northern KwaZulu-Natal (Brooke *et al.*, 2015). This species' inherent resilience places immense pressure on the goal to eliminate malaria by 2030 (World Health Organization, 2019). Therefore, alternative and additional tools to supplement the current vector control methods are needed to reduce local malaria transmission.

The opportunistic nature of *An. arabiensis* presents an additional opportunity to target this malaria vector, as *An. arabiensis* readily feeds on animals as well as human hosts (Gillies and Coetzee, 1987). Consequently, zooprophylaxis could be applied in combination with other vector control strategies. Agricultural and domestic animals are not hosts of human malaria therefore, they could be used to divert *An. arabiensis* away from human hosts through active or passive zooprophylaxis (Habtewold *et al.*, 2004; Mahande *et al.*, 2007).

Simultaneously, the *An. arabiensis* vector population could be targeted when feeding on these hosts, as agricultural animals are frequently vaccinated with recombinant proteins to control parasitic infestations (de la Fuente *et al.*, 1998; Rodríguez *et al.*, 2004; Moreno-Cid *et al.*, 2013; Letinić *et al.*, 2020). Since previous studies have shown efficacy of Subolesin/Akirin ortholog vaccines for the control of ectoparasite infestations, targeting Akirin in *An. arabiensis*, while feeding on vaccinated hosts, could serve as a supplementary intervention to the current vector control strategies.

## 1.3 Subolesin/Akirin

Recombinant vaccines are frequently used for the control of multiple endo- and ectoparasitic infestations in agricultural animals (de la Fuente *et al.,* 1998; Rodríguez *et al.,* 2004; Moreno-Cid *et al.,* 2013). These vaccines contain protective antigens that are expressed from target arthropods. When agricultural animals are immunised with these recombinant vaccines, antigen-specific antibodies develop in the immunised hosts (Willadsen *et al.,* 1989). The arthropod vectors ingest these antibodies when taking a blood meal from the immunised hosts.

The ingested antibodies interact with and disrupt the function of the target antigen in the arthropod, resulting in physiological changes that affect the vector's biology (Elvin and Kemp, 1994). *Anopheles arabiensis* have a marked preference for blood-feeding from cattle (Mutero *et al.,* 2004) therefore, a recombinant vaccine containing a protective antigen expressed from *An. arabiensis* could be used to target the exophagic vector population. However, the development of these recombinant vaccines is limited by the discovery of such antigens (de la Fuente and Kocan, 2003).

A protective antigen, initially called 4D8, was found in *Ixodes scapularis*, using expression library immunisation (Almazán *et al.,* 2003). This antigen was later called Subolesin, after its function was characterised using a series of knockdown experiments (de la Fuente *et al*., 2006). Immunisation of various agricultural animals, using a recombinant Subolesin vaccine, caused several deleterious effects in engorged ticks, including that of diminished vectorial capacity, reduced oviposition, and reduced survivorship (Almazán *et al.,* 2005; Almazán *et al.,* 2010). Feeding capabilities were also significantly reduced, resulting in a decline of tick mass (de la Fuente *et al.,* 2006). Furthermore, developmental abnormalities were observed, such as the failure of nymph metamorphosis, as well as tissue damage in various tick species (Almazán *et al.,* 2005; de la Fuente *et al.,* 2010). Through phylogenetic analysis, tick Subolesin was found to have a high degree of sequence and functional homology to Akirin, which is a nuclear protein found throughout the metazoan world (Sutterwala and Flavell, 2008; Galindo *et al.,* 2009).

Akirin is highly conserved at the N-terminal and the C-terminal domains. These domains are separated by a stretch of less conserved residues, which accounts for the variation in size and sequence similarity amongst various species. Akirin generally ranges from 180 to 210 residues in size, and is strictly localised in the nucleus (Goto *et al.,* 2008). Vertebrates have two closely

related Akirin homologues, namely Akirin1 and Akirin2, while invertebrates have only one copy of Akirin (Galindo *et al.*, 2009; Macqueen and Johnston, 2009). When the function of Akirin1 and Akirin2 were tested in vertebrates, Akirin1 was shown to be essential in skeletal muscle cell differentiation, skeletal myogenesis, and myotube formation (Chen *et al.*, 2013), whereas Akirin2 was crucial for embryogenesis (Goto *et al.*, 2008; Macqueen and Johnston, 2009).

In *Drosophil*a, the generation of Akirin null mutants resulted in several lethal phenotypes characteristic of defective muscle patterning, including improper muscle attachment, muscle duplication, and muscle absence (Goto *et al.*, 2008). Akirin was therefore required during embryogenesis for the expression of genes that are regulated by Twist, which is a transcription factor needed for mesodermal and skeletal musculature development (Goto *et al.*, 2008; Nowak *et al.*, 2012). The Akirin homologue identified in invertebrates (GenBank: NM_001274582) was evolutionarily and functionally closer to that of the Akirin2 (GenBank: NM_018064) (Goto *et al.*, 2008), with an amino acid sequence similarity of 58.5%.

Additionally, both invertebrate Akirin and vertebrate Akirin2 were found to regulate the expression of a subset of genes together with, or downstream of, NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B-cells) (Goto *et al.*, 2008). Since Akirin shows no apparent DNA or RNA-binding motifs, it is improbable that Akirin regulates transcription by binding to DNA directly. Instead, Akirin acts as a co-factor that regulates transcriptional activity by interacting with components of chromatin or the transcriptional machinery (Goto *et al.*, 2008). When transcription takes place, by RNA polymerase II, an initiation complex is assembled. The initiation complex is controlled by the incorporation of promoter DNA into the nucleosomes and other chromatin structures (Goto *et al.*, 2008). This allows transcription to be modulated by chromatin remodelling co-factors which target the nucleosomes, or general co-factors that associate with the basal transcription machinery. Therefore, Akirin acts as a nuclear transcription co-factor that regulates NF-κB activity. This subsequently causes Akirin to play a crucial role in immunity (Goto *et al.*, 2008).

Like all invertebrates, insects lack an adaptive immune system, thus they depend entirely on the innate immune system for protection against pathogens (Stephens, 1959). The innate immune system consists of two distinct signalling pathways, namely the Toll pathway and the immune deficiency (IMD) pathway (Lemaitre *et al.*, 1995; Hoffmann *et al.*, 1996). After

ingesting a blood meal, the midgut bacterial levels increase rapidly. This causes an increase in bacterial elicitors, such as meso-diaminopimelic acid type peptidoglycans (DAP-PGNs) (Stephens, 1959; Kumar *et al.*, 2010). The DAP-PGNs bind to the transmembrane peptidoglycan recognition protein (PGRP), known as PGRP-LC, which initiates the IMD pathway (Figure 1.1) (Choe *et al.*, 2002).

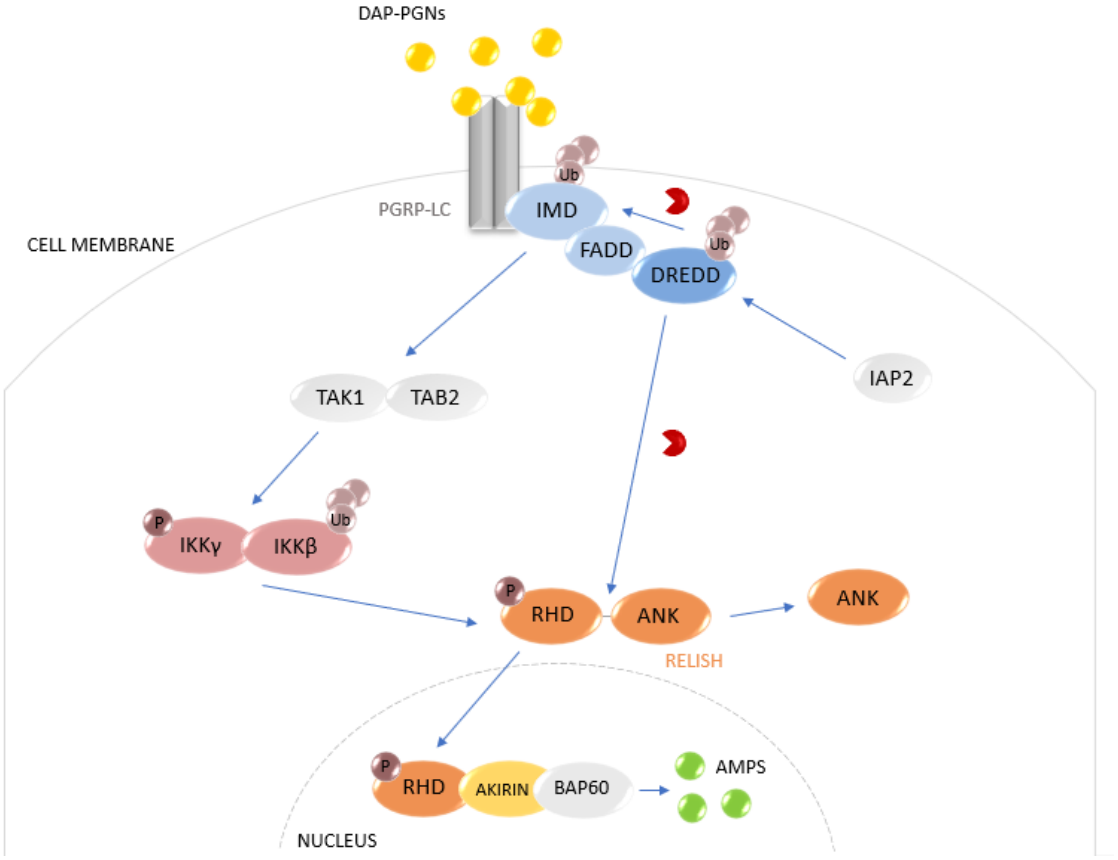


**Figure 1.1** The activation of the IMD pathway results in the translocation of IMD, FADD (Fas-associated protein with death domain), and DREDD (death-related ced-3/nedd2-like protein) from the nucleus to the plasma membrane (Gottar *et al.*, 2002). DREDD is polyubiquitinated (Ub) by the protein IAP2 (inhibitor of apoptosis 2) (Vaux and Silke, 2005). This causes DREDD to cleave IMD, which exposes the Lysine (K63) site for polyubiquitination. Polyubiquitination of K63 causes TAK1 (TGF-β activated kinase 1) to form a complex with TAB2 (TAK1-binding protein1). This complex phosphorylates the IKK (inhibitor of κB kinase) subunits (IKKβ and IKKγ) (Paquette *et al.*, 2010), which allows for phosphorylation, and DREDD-mediated cleavage, of the NF-κB-like transcription factor, Relish (Ertürk-Hasdemir *et al.*, 2009). Relish is made up of the Rel homology domain (RHD) and the inhibitory ankyrin-repeat rich domain (ANK) (Dushay *et al.*, 1996). Cleavage of the ANK domain allows RHD to translocate into the nucleus, where it binds to complex formed by Akirin and BAP60. This allows for the synthesis of several antimicrobial peptides (AMP) (Goto *et al.*, 2008; Sutterwala and Flavell, 2008). Figure adapted from Zakovic and Levashina *et al.* (2017).

Activation of the IMD pathway synthesizes AMPs, such as Attacin and Diptericin (Goto *et al.,* 2008; Bonnay *et al.,* 2014), which reduce the number of microbiotas in the mosquitoes' midgut back to the basal level (Dong *et al.,* 2009; Meister *et al.,* 2009). Therefore, *akirin* knockdown impairs the gene expression of the AMPs, which may disrupt bacterial homeostasis (Mukherji *et al.,* 2013; Debalke *et al.,* 2019). This weakens the innate immune defence, causing enhanced sensitivity to Gram-negative bacterial infection, which subsequently affects vector survival (Ferrandon *et al.,* 2007; Goto *et al.,* 2008; Nowak *et al.,* 2012; Bonnay *et al.,* 2014).

Mosquito longevity is a critical factor affecting malaria transmission. A female mosquito must survive longer than the extrinsic incubation period to transmit the *Plasmodium* parasite (Ohm *et al.,* 2018). Therefore, for a female mosquito to transmit malaria, the mosquito must ingest a blood meal infected with gametocytes. The ingested parasites must encounter the midgut epithelium, develop into gametes, undergo fertilization, form zygotes, develop into motile ookinetes, transverse across the midgut epithelium, evade the mosquito's immune responses, and develop into oocysts (Sinden, 1984). The oocysts must then undergo several rounds of replication to produce haploid sporozoites. The sporozoites must be released into the mosquito hemocoel, migrate through the haemolymph, and invade the salivary glands, to be injected into the next vertebrate host when taking a successive blood meal (Sinden, 1984). This process takes 10 to 14 days to complete (Levashina, 2004; Vlachou *et al.,* 2006; Aly *et al*., 2009).

The pleiotropic nature of Akirin/Subolesin makes immunisation of agricultural hosts, with this protective antigen, a viable option for *An. arabiensis* vector control. *Anopheles arabiensis* that have taken a gametocyte infected blood meal usually take several additional blood meals before completing the extrinsic *P. falciparum* incubation period (Debalke *et al.,* 2019). The repeated ingestion of anti-Akirin/ anti-Subolesin antibodies would neutralise the function of Akirin, subsequently causing detrimental effects within the vector, including a reduction in survival and reproductive capacities. A reduction in *An. arabiensis* survivorship, as well as reproductive fitness, would ultimately cause a decrease in the vector population. A reduction in the vector population would have a significant impact on residual malaria transmission in countries like South Africa (Killeen, 2014; Killeen *et al.,* 2016).

Recombinant Akirin antigens have, however, previously been assessed on several other Culicidae species with variable results (Canales *et al.,* 2009; Moreno-Cid *et al.,* 2010; Moreno-

Cid *et al.,* 2013; da Costa *et al.,* 2014). Therefore, a better understanding of Akirin's function in this malaria vector is needed, to determine whether targeting Akirin is a viable option for *An. arabiensis* vector control. The biological function of Akirin was attested in a colonised strain of *An. arabiensis* mosquitoes that were originally collected in Mamfene, northern KwaZulu-Natal, using man baited nets (Hargreaves *et al.,* 2003). Specific focus was given to the effect of *akirin* knockdown on *An. arabiensis* fecundity, fertility, and longevity. This was done by conducting two different expression suppression techniques, namely, RNA mediated interference (RNAi), as well as the novel gene suppression technique, CRISPR interference (CRISPRi).

RNA mediated interference is a well-established technique, that is administered to a diversity of insect orders, for the characterisation of biological gene functions. This technique uses exogenous double-stranded RNA (dsRNA) to suppress the expression of endogenous messenger RNA (mRNA), causing a subsequent loss of gene transcript (Fire *et al.,* 1998). Similarly, CRISPRi also results in a loss of gene transcript, although this technique is designed to block transcription by preventing transcriptional elongation (Ghosh *et al*., 2016), hence suppressing gene expression. CRISPRi is considered a more predictable and precise tool for silencing genes (Larson *et al*., 2013) however, it is still a novel technique that has yet to be performed in Culicidae. Therefore, both RNAi and CRISPRi were used to repress *akirin* expression, thus allowing for the biological function of this nuclear transcription co-factor to be established in *An. arabiensis*.

Once the biological function of Akirin was assessed within *An. arabiensis* (Letinić *et al.,* 2020), this protective antigen was used to develop a recombinant Akirin vaccine. This vaccine was subsequently evaluated on the colonised *An. arabiensis* vector population, using rodent immunisation trials. Additionally, the recombinant vaccines that were previously tested on the various other Culicidae species were also evaluated on this vector population (Moreno-Cid *et al.,* 2010; Moreno-Cid *et al.,* 2013; da Costa *et al.,* 2014). This was done to determine whether targeting Akirin in *An. arabiensis* could serve as an additional vector control intervention for the untargeted exophagic vector population.

## 1.4 Aim and Objectives

This study aimed to attest to the biological function of Akirin in *Anopheles arabiensis* lifetable parameters, and determine its viability as a potential target for vector control.

**The following objectives, with sub-objectives, were used to achieve the aim:**

1) A protocol was established and executed to repress *akirin* transcription, in *An. arabiensis,* using CRISPR mediated interference (chapter 3: CRISPR interference).

   - A sgRNA was synthesised and combined with a nuclease-deficient form of the Cas9 protein to perform CRISPRi inoculation, in *An. arabiensis,* using nano-injection.
   - The relative gene expression of *akirin* was determined in *An. arabiensis* post-CRISPRi inoculation.
   - The effect of *akirin* suppression, by CRISPRi, was evaluated on *An. arabiensis* longevity.

2) The effect of *akirin* knockdown was determined on *An. arabiensis* lifetable parameters, using RNA mediated interference (chapter 4: RNA interference).

   - Akirin-specific dsRNA was i*n vitro* transcribed to target Akirin mRNA in *An. arabiensis,* when performing RNAi by nano-injection.
   - The relative gene expression of *akirin* was determine in *An. arabiensis,* post-RNAi inoculation.
   - The effect of *akirin* suppression, by RNAi, was evaluated on *An. arabiensis* lifetable parameters.

3) The effect of recombinant Akirin vaccines was assessed on *An. arabiensis,* to determine its potential as a vector control tool (chapter 5: Immunisation).

   - Recombinant Akirin vaccines were formulated.
   - Recombinant Akirin immunisation was performed on balb/c mice, and assess the antibody titres.
   - The effect of ingesting anti-Akirin antibodies was evaluated on *An. arabiensis* lifetable parameters.

# CHAPTER 2: COMMON METHODS

## 2.1 Biological Material

A laboratory strain of *An. arabiensis* mosquitoes (MBN) was used in this study. This strain was colonised in 2002, from wild material that was collected in Mamfene, northern KwaZulu-Natal, South Africa (Hargreaves *et al.*, 2003). The colony is maintained in the Botha De Meillon Insectary, at the Vector Control Reference Laboratory (VCRL) of the National Institute for Communicable Diseases (NICD) in Johannesburg, under standard insectary conditions of 80% (+/- 5%) humidity, 25°C (+/- 2˚C), and a 12-hour day/night cycle with 45-minute dusk/dawn transitions (Hunt *et al*., 2005). It is sustained on a diet of finely ground Beeno® dog biscuits and brewer's yeast (3:1) for the aquatic larval life stages, and a 10% sucrose solution for the terrestrial adult life stages (Hunt *et al*., 2005). The female mosquitoes are provided with a blood meal twice a week via routine guinea pig blood feeding.

The guinea pig colony is maintained at the National Health Laboratory Service (NHLS) animal unit, in standard laboratory cages, until issued. The original breeding stock for this colony was bought from Harlan Laboratory in the United Kingdom. Guinea pigs are kept at a temperature range of 21˚C (+/- 2˚C), with a light/dark cycle of 12-hours. They are sustained on a diet of commercial rabbit pellets (Epol®, 11108) and water *ad-lib*. They are provided with a bed of pine shavings, with added *Eragrostis*, which is changed three times per week. To prevent unnecessary distress, guinea pigs are anaesthetised prior to blood-feeding. All animal handling and veterinary procedures are strictly conducted by trained South African Veterinary Council (SAVC) registered staff. The routine blood-feeding protocol and ethics were reviewed and approved by the NHLS, Animal Ethics Committee (AEC) (AESC: 1993–047). See Appendix C.

## 2.2RNA Isolation

RNA isolation was performed using TRI reagent (Sigma-Aldrich, T9424), as per the manufacturer's specifications. Ten *An. arabiensis* mosquitoes (60mg) were homogenized in 600µL of TRI reagent. The homogenized solution was incubated at room temperature for 5-minutes. Chloroform was added to the solution (1:5), and mixed by inversion, after which the samples were incubated at room temperature for an additional 5-minutes. The samples were centrifuged at 12,000 x *g* (15-minutes, 4˚C). This was done to induce phase separation, causing the samples to separate into three separate phases namely, a red organic protein-containing phase, an interphase containing DNA, and a colourless upper aqueous phase containing RNA. The upper aqueous phase was transferred into a new micro-centrifuge tube, where the RNA was precipitated by adding isopropanol (300µL) to the solution, which was mixed via inversion. The samples were incubated at room temperature for 10-minutes. Centrifugation was conducted at 12,000 x *g* (10-minutes, 4˚C), to allow an RNA pellet to form at the bottom of the tube. The supernatant was discarded, and the pellet was washed with 600µL of 75% ethanol. The samples were centrifuged at 7,500 x *g* (5-minutes,4˚C), and the ethanol was removed. The pellet was left to air dry for 10-minutes and re-suspended in 30µL of RNase-free water. DNase I (NEB, M0303S) treatment was conducted by adding DNase I (2U) and 3x reaction buffer to the re-suspended samples. The samples were then incubated for 15-minutes at room temperature. The enzyme was heat-inactivated by incubating the samples at 70˚C for 15-minutes. The NanoDrop™ spectrophotometer was used to assess the concentration of the isolated RNA samples.

## 2.3 RNA Purification

The isolated RNA samples were purified using the RNeasy® MinElute™ clean-up kit (Qiagen, 74204). Purification was conducted to remove residual salts and other impurities from the samples. RNase-free water was used to adjust the samples to a final volume of 100µL. Lysis buffer (350µL) was added to the diluted RNA samples and mixed thoroughly using the vortex. To promote selective binding of the RNA to the RNeasy® MinElute™ membrane, 100% ethanol (250µL) was added to each sample. The samples were mixed by pipetting and transferred to the RNeasy® MinElute™ spin column, which was placed in the provided collection tubes. The samples were centrifuged for 15-seconds at 8,000 x *g*. The flow-through was discarded, and the spin columns were transferred into new collection tubes. Wash buffer (500µL) was

transferred to the spin column, after which centrifugation was conducted at 8,000 x *g* for 15-seconds. The flow-through was discarded, and 80% ethanol (500µL) was added to the spin columns. The tubes were centrifuged for 2-minutes at 8,000 x *g*. The samples were centrifuged at 10,000 x *g* for a further 5-minutes, with the spin column lids open, to ensure that ethanol was not carried over during elution. The flow-through and collection tubes were discarded, and the columns were transferred to new collection tubes. RNase-free water (14µL) was pipetted directly onto the centre of the silica-gel membrane. The samples were then centrifuged for 1-minute at 10,000 x *g,* to elute the RNA. The NanoDrop™ spectrophotometer was used to assess the concentration of the isolated RNA samples.

## 2.4 RNA Native Gel

The integrity of the isolated RNA samples was assessed using a native RNA gel. The RNA native gel was conducted using a 1% agarose, 0.5x TBE (Tris/Borate/EDTA) gel. The samples (1µg) were prepared using a 1:1 ratio of sample to RNA loading dye (ThermoFisher Scientific, R0641). The molecular weight marker dye (ThermoFisher Scientific, SM1821) and the samples were heat-denatured at 70ºC for 10-minutes, after which they were immediately placed on ice. Heat denaturation was conducted to ensure the separation of the ribosomal proteins when performing electrophoresis. Electrophoresis was conducted at 70V for 30-minutes, to ensure minimal RNA degradation.

## 2.5 Bioanalyzer

The quality of the RNA samples was assessed using the Agilent 2100 Bioanalyzer system. The RNA 6000 Pico Kit (Agilent, G2938-90049) was used to generate an RNA integrity number (RIN) for each sample, following the manufacturer's instructions. The RNA ladder and samples were heat-denatured at 70°C for 2-minutes before being loaded on the pico-chip. The marker was loaded into all 12 sample wells (5µL). The prepared ladder (1µL) was loaded into the well-marked "", and the prepared samples (1µL) were loaded into the 12 sample wells. The pico-chip was then placed into the chip adapter of the vortex, and vortexed for 1-minute at 2,400 x *g*. The pico-chip was then placed in the bioanalyzer and electrophoresed within 5-minutes of preparation. A RIN value ranging between 3-5 is indicative of good quality RNA for insects (Agilent Technologies, 2017).

## 2.6 Complementary DNA Synthesis

Complementary DNA (cDNA) was synthesized from the isolated RNA. This was done using the QuantiTect® reverse-transcription kit (Qiagen, 205311), as per the manufacturer's specifications. Genomic DNA was eliminated by adding 1x genomic DNA wipe-out buffer to 1µg of RNA sample, which was made up to a final volume of 14µL using RNase free water. The samples were subsequently incubated at 42°C for 2-minutes, to allow for adequate genomic DNA elimination. Reverse-transcription was conducted by adding Quantiscript® reverse-transcriptase, reverse-transcription primer mix, and 5x Quantiscript® reverse-transcription buffer, in a 1:1:4 ratio, to the RNA. The samples were incubated at 42°C for 15-minutes, to allow for cDNA synthesis to take place. The Quantiscript® reverse-transcriptase was subsequently inactivated by incubating the samples at 95°C for 3-minutes. The NanoDrop™ spectrophotometer was used to assess the concentration of the cDNA samples.

## 2.7 Longevity Assessment of Mosquitoes

Survival analysis was performed in 32.5cm$^3$ insect rearing cages (BugDorm®, 211476). The *An. arabiensis* used to assessed vector longevity were sustained on a 10% sucrose solution diet for the duration of the experiment. The rate of *An. arabiensis* survival was monitored until 100% mortality was attained for all treatments. A Kaplan-Meier survival curve was constructed, using a statistical program (Statistix 10), to assess the rate of *An. arabiensis* survival between the various treatments. The probability of survival for each treatment was displayed as horizontal lines on the survival-curve, while the change in cumulative probability was displayed as vertical lines (Kaplan and Meier, 1958). The cumulative probability changed when a death occurred (Rich *et al.*, 2010). The Log-rank test (Bland and Altman, 2004) was used to determine whether there was a statistically significant difference in vector longevity between the various treatments (Log-rank: $p<0.05$).

# CHAPTER 3: CRISPR INTERFERENCE

**Objective:** Establish and execute a protocol to repress *akirin* transcription, in *An. arabiensis*, using CRISPR mediated interference.

The optimised nano-injection protocol described in Chapter 3 and 4 was published on 1 September 2018 (Appendix D): **Letinić, B.D., Kemp, A., Christian, R.N. and Koekemoer, L.L.**, 2018. Inoculation protocol for the African malaria vector, *Anopheles arabiensis*, by means of nano-injection. *African Entomology*, *26*(2), pp.422-428.

## 3.1 Introduction

Genome editing technologies allow for the easy alteration of DNA sequences and modification gene functions. Clustered regularly interspaced short palindromic repeat (CRISPR) is a genome editing technology that was initially discovered in *Escherichia coli* as an array of short identical direct repeats (29bp), that were separated by variable spacer regions (32nt) (Ishino *et al.*, 1987). The same loci were later reported in numerous prokaryotic species and were subsequently named CRISPRs (Jansen *et al.*, 2002). Additionally, CRISPR-associated (Cas) genes were detected in close proximate to the CRISPR loci (Jansen *et al.*, 2002).

When the spacers in the CRISPR loci were sequenced, they were found to share sequence homology with both phages and plasmids (Mojica *et al.*, 2005; Pourcel *et al.*, 2005). The extrachromosomal origin of the CRISPR spaces suggested the possibility of a naturally occurring defence mechanism within prokaryotes, post-infection (Bolotin *et al.*, 2005). The first experimental evidence of this CRISPR-Cas adaptive immunity was shown in *Streptococcus thermophilus*, as novel short pieces of foreign DNA (spacers) were attained in the bacterial genome following phage exposure (Barrangou *et al.*, 2007). These novel spacers could provide phage resistance in a Cas-dependent manner (Barrangou *et al.*, 2007).

CRISPR immunity is performed in three stages, namely the adaptation (spacer acquisition), expression, and interference stage (Brouns *et al.*, 2008). However, CRISPR immunity is facilitated by three different CRISPR-Cas systems (types I, II, and III) (Makarova *et al.*, 2011). During the expression stage, CRISPR repeat-spacer arrays are transcribed into long transcripts known as precursor CRISPR RNAs (pre-crRNAs), that are subsequently processed into short

CRISPR RNAs (crRNAs) (Deltcheva *et al.,* 2011). The type I and type III CRISPR-Cas systems cleave pre-crRNAs to produce mature crRNA, using an endonuclease known as Cas6. The type II CRISPR-Cas system, however, uses a unique trans-activating CRISPR RNA (tracrRNA) molecule to produce mature crRNA (Deltcheva *et al.,* 2011).

In the interference stage, the mature crRNA binds with the Cas protein to form an effector ribonucleoprotein complex (Brouns *et al.,* 2008). The effector complex subsequently recognises and binds to the target sequence of the invasive nucleic acid, and causes sequence-specific cleavage (Brouns *et al.,* 2008). This inhibits additional proliferation and propagation of foreign genetic elements, thus displaying the existence of an adaptive immune defence mechanism analogous to that of the eukaryotic RNAi system (Makarova *et al.,* 2006). The type I CRISPR-Cas system incorporates the crRNAs into a multi-subunit effector complex known as Cascade (CRISPR-associated complex for antiviral defence) (Sinkunas *et al.,* 2011). Cascade binds to the target DNA and causes degradation of the target sequence (Beloglazova *et al.,* 2011; Westra *et al.,* 2012). The type III CRISPR-Cas system uses an effector complex of Cas RAMP proteins (CMR), and crRNAs, to recognise and cleave the target nucleic acid (Hale *et al*., 2012; Zhang *et al.,* 2012).

The type II CRISPR-Cas system, however, uses a single Cas9 protein to induce DNA silencing (Sapranauskas *et al.,* 2011). In the effector complex, Cas9 is bound to both the crRNA and tracrRNA in a ternary complex, which causes cleavage of the invasive nucleic acid (Jinek *et al.,* 2012). The type II effector complex, therefore, functions as an RNA-guided endonuclease, that accomplishes target sequence recognition using crRNA, and uses Cas9 for DNA cleavage within the target protospacer (Jinek *et al.,* 2012). This process provided the impetus for the development of the novel genome-editing tool known as CRISPR, which redirected Cas9 to cleave any region of DNA by changing the nucleotide sequence of the crRNA (Gasiunas *et al.,* 2012). This was further simplified by fusing the crRNA and tracrRNA together to produce a single-guide RNA (sgRNA), which has been shown to function in both bacterial and eukaryotic cells (Jinek *et al.,* 2012; Jinek *et al.,* 2013).

The CRISPR type II system requires only two components for genome editing, namely a custom-designed sgRNA and a Cas9 protein (Jinek *et al.,* 2013). The sgRNA is created by designing a 20bp oligonucleotide that is complementary to a selected target region in the gene of interest. This 20bp oligonucleotide (crRNA) is then fused to a 122bp oligonucleotide

(tracrRNA) (GenBank: KX446948.1) to form a stem-loop structure, which binds to the Cas9 protein (Kistler *et al.*, 2015). The sgRNA directs the Cas9 protein to the complementary target in the gene of interest, and binds to it if an adjacent 3bp protospacer-adjacent motif (PAM) ($^{5'}$NGG$^{3'}$) is present on the non-targeted DNA strand (Jinek *et al.*, 2013; Larson *et al*., 2013).

Binding of the Cas9 protein to the selected target region mediates cleavage of the target sequence by two nuclease domains, RuvC1 and HNH, causing a double-strand break in the target sequence (Larson *et al*., 2013; Ghosh *et al*., 2016) (Figure 3.1). This double-strand break is repaired using either non-homologous end-joining (NHEJ) or homology-directed repair (HDR). NHEJ is used when knocking out a target DNA sequence, while HDR is used to insert a sequence of interest or introduce a mutation (Sander and Joung, 2014). This generates changes at the genomic level, which are both stable and heritable, and can consequently be transmitted to the next generation (Perkin *et al.*, 2017).

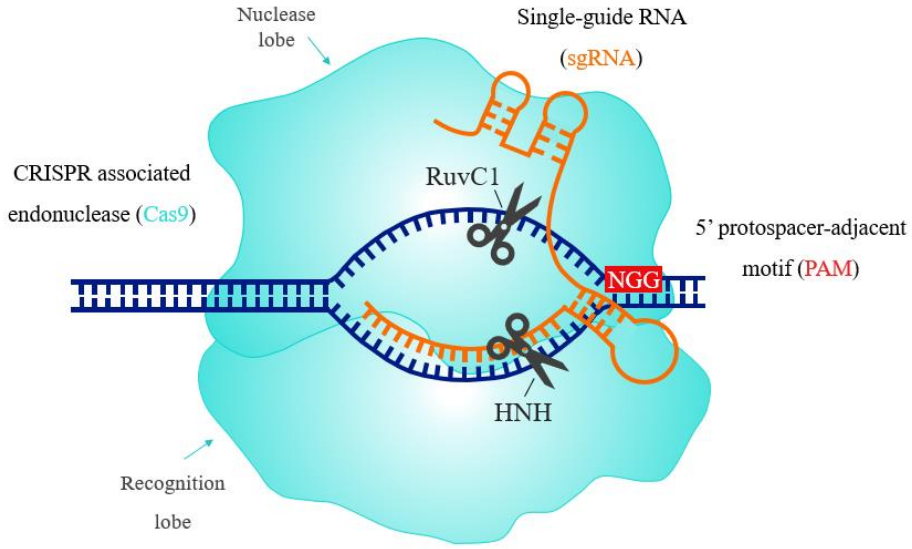


**Figure 3.1** The CRISPR system used to silence gene expression, by the direct pairing of the sgRNA (orange) and the Cas9 protein (light blue), which has endonuclease activity due to the RuvC1 and HNH nuclease domains, to a specific target site within the gene of interest (dark blue), located adjacent to the PAM sequence (red). Figure adapted from Dominguez *et al*. (2016).

The CRISPR–Cas9 type II system has subsequently been repurposed as a sequence-specific gene regulation tool called CRISPR interference (CRISPRi). This was done by introducing two respective point mutations in the Cas9 protein, at the RuvC1 (D10A) and HNH (H840A) nuclease domains, resulting in a nuclease-deficient Cas9 (dCas9) protein (Qi *et al*., 2013; Larson *et al*., 2013; Ghosh *et al*., 2016). These mutations prevent cleavage of the target DNA

from taking place however, dCas9 still retains its ability to recognise and bind to the target DNA, using the sgRNA (Qi *et al*., 2013).

A sgRNA designed to target the promoter region of the target gene causes steric hindrance between the key *cis*-acting DNA motifs and their cognate *trans*-acting transcription factors (Qi *et al*., 2013). The transcription factors generally form a complex with RNA polymerase II, which binds to the transcriptional start site (TSS), allowing for transcription to take place (Nikolov and Burley, 1997). Steric hindrance of the transcription factors would prevent the recruitment of RNA polymerase II, which in turn, would lead to the repression of transcription initiation (Figure 3.2). Alternatively, a sgRNA designed to target a downstream site, within the target gene, creates a physical barrier that blocks transcription elongation (Qi *et al*., 2013).

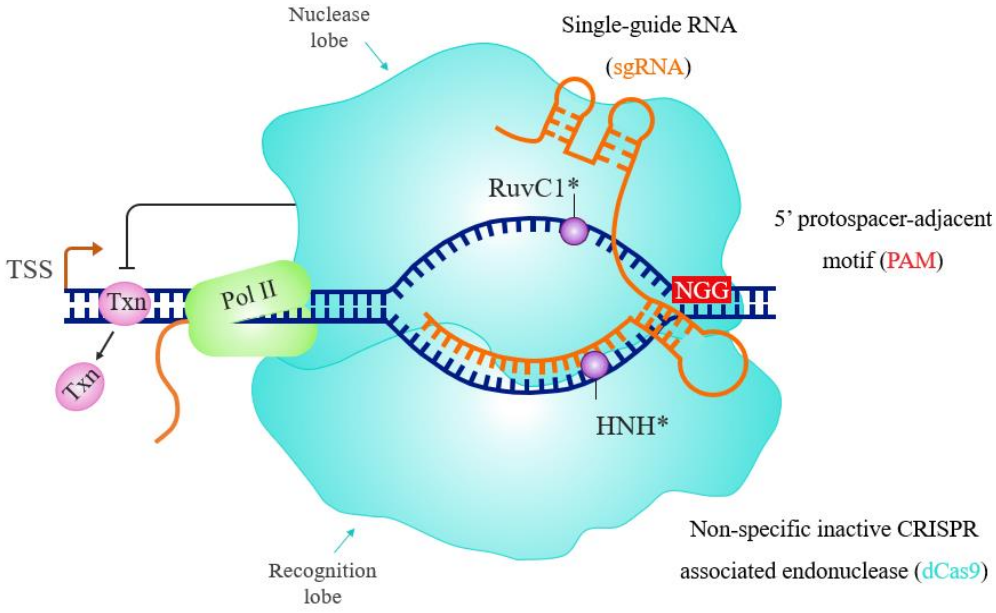


**Figure 3.2** The adapted CRISPRi system used to repress transcription (Txn), through the steric hindrance of the transcription start site (TSS), caused by the binding of the sgRNA-dCas9 complex to the target sequence. Figure adapted from Dominguez *et al*. (2016).

The suppression of transcription initiation or transcription elongation ultimately results in the downregulation of gene expression. CRISPR interference allows for the direct manipulation of the transcription process without genetically modifying the DNA sequence. Therefore, unlike the perpetual genetic modifications induced by CRISPR, gene repression using CRISPRi is reversible (Qi *et al*., 2013), following the termination of dCas9 expression. The CRISPRi system was, therefore, used to regulate *akirin* expression in *An. arabiensis* on a transcriptional level.

Regulating gene expression on a transcriptional level provided insight into the function of Akirin in the biological pathways of this malaria vector. This was, however, a novel technique, that had not yet been performed on Culicidae. Previously, the CRISPR–Cas9 type II system had been used to generate site-specific mutations in *Ae. aegypti*, which is a vector of other mosquito-borne diseases such as chikungunya, dengue virus, and yellow fever (Kistler *et al.*, 2015). Since CRISPRi is an adapted form of the CRISPR-Cas9 type II system, this gene regulation technique was performed in *An. arabiensis* using the nuclease-deficient form of the Cas9 protein.

The dCas9 protein was administered to female *An. arabiensis* mosquitoes, along with a custom-designed sgRNA, using nano-injection. The sgRNA was designed to target a specific site within the first exon of Akirin. This allowed dCas9 to bind to the Akirin DNA sequence in *An. arabiensis*, through direct pairing. The binding of dCas9 would result in the prevention of transcription elongation, which would cause a decrease in *akirin* expression. A sgRNA designed to target Mouse Beta-2 microglobulin (β2m) was used as a negative control (Fernández-Verdejo *et al.*, 2017). This is a protein found within *Mus musculus*, which does not have a complementary sequence within the *An. arabiensis* genome. Therefore, no phenotypic or genotypic effect was observed when the sgRNA: dCas9 complex was administered to *An. arabiensis* using nano-injection.

The downregulation of *akirin* expression was attested in *An. arabiensis* by performing reverse-transcription quantitative PCR (RT-qPCR). *Anopheles arabiensis* survival was thereafter assessed to determine whether *akirin* knockdown affected vector longevity. Akirin is a vital component of various biological pathways including the IMD signalling pathway, which is needed for defence against bacterial infection (Ferrandon *et al.*, 2007; Goto *et al.*, 2008). Consequently, repressing *akirin* expression would have an impact on vector survival, which is a crucial factor in malaria transmission.

The effects of *akirin* knockdown were subsequently assessed on *An. arabiensis* longevity using CRISPRi. These results were used to determine whether targeting Akirin could be further developed as an additional tool to reduce the mean survival time of the *An. arabiensis* vector population, which would aid in the reduction of malaria transmission.

## 3.2 Materials and Methods

### 3.2.1 Synthesis and combination of sgRNA with dCas9, to perform CRISPRi inoculation in *An. arabiensis* by nano-injection

The online sgRNA design tool (http://crispr.mit.edu) was used to identify putative sgRNA target sites, within the first exon of the non-template strand, of the Akirin (VectorBase: AARA009142) and Mouse-β2m (GenBank: NM_009735) nucleotide sequences. Once the putative sgRNA target sites were identified, they were incorporated into primers that would be used to generate a sgRNA PCR product (dsDNA), that would subsequently be *in vitro* transcribed into dsRNA (Kistler *et al.*, 2015). The forward primer was designed to contain the sgRNA target sequence (excluding the PAM sequence), which was preceded by a T7 promoter sequence ($^{5'}$TTAATACGACTCACTATA$^{3'}$). The reverse primer used was a universal primer that could be used for all sgRNA templates. These primer sequences are illustrated in Appendix A. The primers were designed in such a way that they overlapped, and were annealed together when conducting a PCR (polymerase chain reaction), to create single sgRNA products (dsDNA) of approximately 122bp (Figure 3.3).

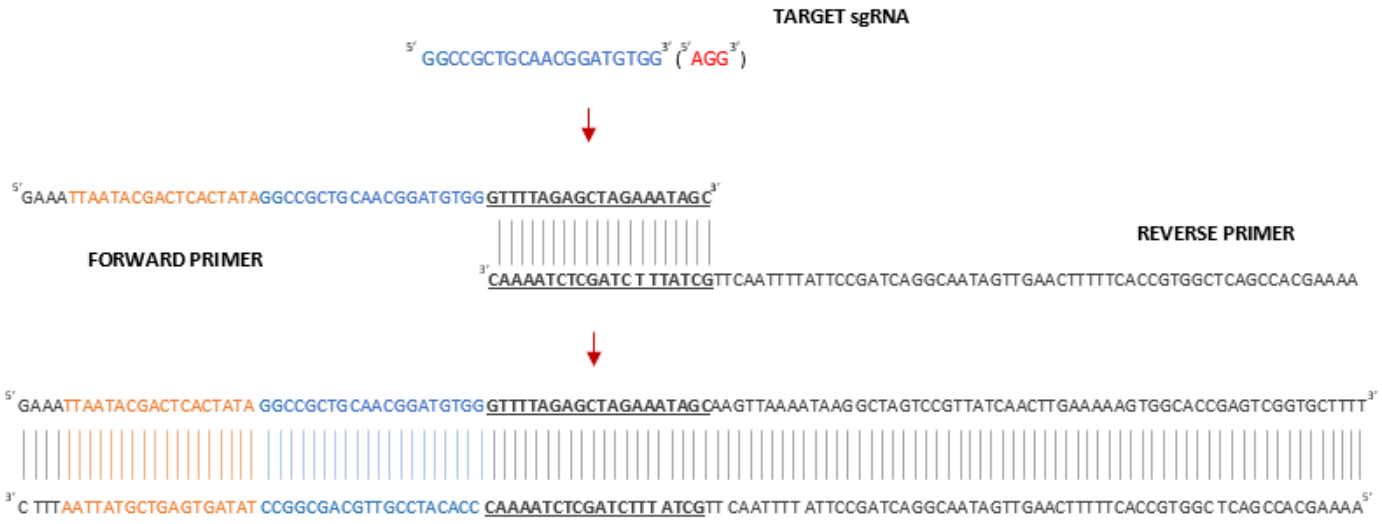


**Figure 3.3** The sgRNA PCR template generated using the forward and reverse primers. A forward primer was designed once a putative sgRNA target site (blue) was identified adjacent to a 3bp PAM sequence (red). The forward primer contained the T7 promoter (orange), which would be used to initiate *in vitro* transcription. This promoter sequence was followed by the sgRNA target sequence. The reverse primer used was a universal sequence, which is used to generate all sgRNA templates. The underlined portion of the two sequences represents a complementary region, which allows the two primers to anneal together when conducting PCR.

The sgRNA forward and reverse primers were amplified, using the TaKaRa Taq™ version 2.0 DNA polymerase premix (TaKaRa, R004A), to synthesize the sgRNA PCR products (dsDNA). The PCR was optimised using 1x PCR buffer (10mM Tris-HCl, 5mM KCl), 1mM dNTP's (0.25mM each), 1.5mM $MgCl_2$, 0.5µM forward primer, 0.5µM reverse primer, and 0.1U *Taq* (*Thermus aquaticus*) polymerase, which was made up to a final volume of 100µL using nuclease-free water, for both sgRNA templates. The PCR reaction was performed by preheating the reaction to 95°C for 2-minutes, followed by 40 PCR cycles (20-seconds at 95°C, 10-seconds at 58°C, 10-seconds at 72°C). The size and specificity of the synthesized oligonucleotides were assessed using a 2.5% agarose gel in TAE (Tris/acetate/EDTA), which was electrophoresed for 75-minutes at 100V, using 200ng of sgRNA PCR product (dsDNA).

The sgRNA PCR products (dsDNA) were purified using AMPure® beads (Beckman Coulter, A63880) according to the manufacturer's recommendations. The AMPure® beads were added to the sample using a 1:1 ratio. The solution was mixed using the vortex, after which the samples were incubated at room temperature for 5-minutes. The samples were placed on a magnetic stand until the solution became clear. The supernatant was discarded, and the remaining beads were rinsed with 300µL of freshly made 80% ethanol. The ethanol was discarded, and the samples were left to air dry for 5-minutes. The samples were removed from the magnetic stand and were resuspended in 30µL of nuclease-free water. The concentration of the purified samples was quantified using the NanoDrop™ spectrophotometer, and sequenced by Macrogen, Netherlands.

The MEGAshortscript™ T7 transcription kit (Life Technology, AM1354) was used to *in vitro* transcribe the sgRNA PCR products (dsDNA), targeting both Akirin and Mouse-β2m, into dsRNA. The 18S (80bp) control template supplied within the MEGAshortscript™ kit was used as a positive control. In a 20µL reaction, 1-fold of ATP, CTP GTP, UTP, T7 enzyme mix, and reaction buffer was added to the 300ng sgRNA DNA template. The T7 promoter region, added by the forward primer, allowed for *in vitro* transcription to be initiated. The reaction was incubated at 37°C for 4-hours. TURBO™ DNase (2U) was added to the reaction, and was incubated for an additional 15-minutes, at 37°C, to digest any residual DNA.

The MEGAclear™ kit (Life Technology, AM1908) was used to purify the *in vitro* transcribed sgRNA, as per the manufacturer's specifications. Elution solution (100µL), binding solution (350µL), and 100% ethanol (250µL) were added to the sgRNA samples. The samples were transferred into the supplied filter cartridges, and centrifuged at 10,000×*g* for 1-minute. The flow-through was discarded. The filter cartridge was washed using the wash solution (500µL), and was centrifuged at 10,000×*g* for 1-minute. After centrifugation, the flow-through was once again discarded. Centrifugation was conducted at 10,000×*g* for an additional 30-seconds, to remove trace amounts of wash solution. The filter cartridge was placed into a new collection tube, where the elution solution (50µL) was added to the centre of the cartridge. The samples were heated to 65°C, for 10-minutes. The purified sgRNA was eluted by conducting centrifugation at 10,000×*g* for 1-minute. Bioanalyzer was performed to assess the quality of the sgRNA samples (see chapter 2, section 5 for detailed method), after which the sgRNA was made up to a final concentration of 3000ng/µL for both Akirin and Mouse-β2m.

Each sgRNA was added to 300ng of commercially purchased dCas9 protein (Novatein Biosciences, PR-137213), to produce two separate CRISPRi inoculation solutions, that would be administered to *An. arabiensis* using nano-injection (Kistler *et al.*, 2015). A nano-injection protocol was established and optimised to administer the CRISPRi inoculation solution to *An. arabiensis* (Letinić *et al*., 2018). The Nanoinject II (Drummond, 3-000204) was used to conduct CRISPRi inoculation on 1-day-old cold anesthetised female *An. arabiensis* mosquitoes, via the thorax, using a volume of 69nL (Appendix D). A subset of cold anesthetised mosquitoes, injected with phosphate-buffered saline (PBS), was used as a handling control, while a subset of mosquitoes that were not subjected to inoculation, but had undergone cold anesthetisation, was used as an untreated control (Letinić *et al*., 2018).

Inoculations were conducted using a total of 75 female mosquitoes per each treatment. Once treated, the female *An. arabiensis* mosquitoes were placed back into the environmentally controlled insectary. Thirty mosquitoes were randomly selected from each treatment for RT-qPCR analysis (10 mosquitoes per replicate, 3 replicates), while the remaining 45 female mosquitoes from each treatment were used to analyse vector longevity (15 mosquitoes per replicate, 3 replicates). All treatments were carried out in 32.5cm$^3$ insect rearing cages (BugDorm®, 211476).

### 3.2.2 Determination of the relative gene expression of *akirin* in *An. arabiensis* post-CRISPRi inoculation

The relative gene expression of *akirin* was determined by performing RT-qPCR analysis. Five putative candidate reference genes were selected to normalise *akirin* expression (*18S, ND5, CO1, S26,* and *S7*). These genes are expressed at a constant level within most tissues, throughout the developmental life stages, without being affected by the experimental treatment (Radonić *et al.*, 2004). Primers were selected to amplify the candidate reference genes as well as *akirin*. The online ThermoFisher primer design tool (https://tools.lifetechnologies.com) was used to design a primer set that would amplify *akirin* in *An. arabiensis,* while primers for the reference genes were selected based on gene regulation analysis, that had been previously optimised and performed in *Anopheles* mosquitoes (Table 3.1). These primers sets were subsequently ordered from Macrogen, Korea.

**Table 3.1** Primers used to assess the relative gene expression when performing RT-qPCR

| Gene | Reference | Primer sequence | Tm (°C) | GC (%) | Amplicon size (bp) |
|---|---|---|---|---|---|
| *akirin* GenBank: KU973613 | N/A | F_5′AGACAGCCCTCCTAGCAT 3′ | 58.0 | 56 | 149 |
| | | R_5′GTTCGGCTAGTTTAGTGGTC 3′ | 57.0 | 50 | |
| *ND5* GenBank: JX117430.1 | Spillings, 2012 | F_5′ TAGAATTTTATTAGGGTGGGATGG 3′ | 55.3 | 38 | 121 |
| | | R_5′ ATCTCCAATTCGATTTGATAATGC 3′ | 54.5 | 33 | |
| *CO1* GenBank: KU187109.1 | Spillings, 2012 | F_5′ TAGGAGCACCTGATATAGC 3′ | 54.3 | 47 | 124 |
| | | R_5′ ACAGTTCATCCTGTTCCAGC 3′ | 58.8 | 55 | |
| *S7* VectorBase: AGAP010592 | Jones *et al.*, 2013 | F_5′ AGAACCAGCAGACCACCATC 3′ | 58.8 | 55 | 149 |
| | | R_5′ GCTGCAAACTTCGGCTATTC 3′ | 56.4 | 50 | |
| *S26* VectorBase: AARA003197 | Spillings, 2012 | F_5′ GATAAGGCGATCAAGAAGTTCG 3′ | 52.9 | 50 | 160 |
| | | R_5′ TACGGACAACCTTCGAGTGG 3′ | 58.4 | 55 | |
| *18S* GenBank: AF417777.1 | Munhenga and Koekemoer, 2011 | F_5′ TACCTGGGCGTTCTACTC 3′ | 55.1 | 57 | 130 |
| | | R_5′ CTTTGAGCACTCTAATTTGTTC 3′ | 50.1 | 36 | |

RNA was isolated and purified three-days post-CRISPRi inoculation. This was done to mimic the experimental procedures conducted when the relative expression of *akirin* was assessed in *An. arabiensis*, using RNAi (Letinić *et al.*, 2020). The concentration of the isolated RNA was assessed using the NanoDrop™ spectrophotometer, while the integrity was assessed using a native RNA gel, and the quality was assessed by Bioanalyzer analysis. The isolated RNA samples were reverse-transcribed into cDNA (See chapter 2, section 2-6, for detailed methods), and the cDNA templates were used to amplify *akirin* as well as the reference genes, using the selected primer sets.

The SYBR® Green (Bio-Rad, 170-8887) fluorescent molecule was used to detect and quantify the accumulated amplified product in real-time, as per the manufacturer's specifications. The cycle at which the fluorescent signal becomes detectable is known as the quantification cycle (Cq). The Cq value for each reaction is determined based on the amount of initial template present. A large amount of template results in a low Cq value, whereas a small amount of template results in a high Cq value.

The annealing temperature, as well as the primer concentration used to amplify *akirin* and the reference genes, were optimised. The annealing temperature was optimised using a gradient range of eight different temperatures, between 50ºC - 65ºC. The primer concentration was optimised using a gradient range of various concentrations (2µM, 2.5µM, 3µM, 3.5µM, and 4.0µM). The annealing temperature and primer concentration were chosen based on the conditions which were optimal for both *akirin* and the reference genes.

A dissociation curve was generated to assess whether there was amplification of secondary products caused due to non-specific binding, primer-dimers, or contaminants. The PCR products were sent to Macrogen for sequencing. The sequence alignments generated were edited using BioEdit and analysed using the basic local alignment search tool (BLAST), by the National Centre for Biotechnology Information (NCBI).

Once optimisation was complete and sequencing was confirmed, NormFinder (Andersen *et al.*, 2004) was used to determine which reference genes were the best candidates for conducting RT-qPCR in relation to *akirin*. NormFinder evaluated the Cq values of the reference genes by calculating a stability value for each reference gene. The two reference genes with the lowest values (highest stability) were selected to assess the relative gene expression of *akirin*.

Quantitative-PCR was conducted using 1x iQ™ SYBR® Green supermix (Bio-Rad, 170-8887), 0.48µM Akirin qPCR forward primer, 0.48µM Akirin qPCR reverse primer, and the cDNA template, which was made up to a final volume of 25µL using nuclease-free water. Each cDNA sample was diluted to a concentration of 100ng/µL. These samples were called "unknowns". A standard curve was prepared using cDNA from the samples treated with Mouse-β2m-specific sgRNA (negative control). The standard curve started at a concentration of 1000ng/µL and proceed with a 2-fold dilution to 7.8ng/µL. All samples were amplified using the C1000™ thermal-cycler, Bio-Rad CFX96 Touch™ real-time PCR detection system. PCR amplification was performed by preheating the reaction to 94°C for 2-minutes, followed by 40 PCR cycles (30-seconds at 94°C, 30-seconds at 59.5°C, 40-seconds at 72°C), and a final PCR extension of 10-minutes at 72°C (Letinić *et al*., 2020).

Once amplified, the unknowns needed to fall within the range of the standard curve to be quantified. An efficiency (E) value was used to determine the quality of the standard curve. Successful RT-qPCR was characterised by having an E-value between 90% to 110%, as well as an $R^2$-value greater than 0.980 for each gene (Bio-Rad Laboratories, 2006). REST© analysis was used to determine the relative gene expression ratio of *akirin*. The relative gene expression levels were normalised using the *S7* and *S26* as reference genes. The mRNA expression of *akirin* in the samples treated with Akirin-specific sgRNA was measured in relation to the mRNA expression of *akirin* in the samples treated with Mouse-β2m-specific sgRNA. The mRNA expression of *akirin*, in the samples treated with Mouse-β2m-specific sgRNA, was also measured in relation to the mRNA expression of *akirin* in the PBS and the untreated samples, in each treatment. This was done to ensure that the results being observed were gene-specific and were not altered by the various treatments (Letinić *et al*., 2020).

### 3.2.3 Evaluation of the effect of *akirin* suppression, by CRISPRi, on *An. arabiensis* longevity

Survival analysis was conducted to determine whether *akirin* knockdown influenced *An. arabiensis* longevity. Longevity was evaluated using a total of 45 female mosquitoes per treatment (15 mosquitoes per replicate, 3 replicates). The *An. arabiensis* were sustained on a 10% sucrose solution diet, while the rate of survival was monitored until 100% mortality was reached for all four treatments. See chapter 2, section 7, for a detailed method on survival analysis.

## 3.3 Results

### 3.3.1 Synthesis and combination of sgRNA with dCas9 to perform CRISPRi inoculation, in *An. arabiensis,* by nano-injection.

The online sgRNA design tool returned four possible putative sgRNAs target sites for Akirin. The sgRNA target site with the highest quality score, and the lowest number of off-target sites, was chosen to target Akirin ($^{5'}$GGCCGCTGCAACGGATGTGG$^{3'}$, AGG). The online sgRNA design tool returned one possible sgRNA target site for Mouse-β2m, ($^{5'}$GGTCGCTT CAGTCGTCAGCA$^{3'}$, TGG). This sgRNA target site has also previously been validated in a mouse model for effective knockdown of Mouse-β2m (Fernández-Verdejo *et al.*, 2017). These selected sgRNA target sites were incorporated into primers that were used to generate templet-free sgRNA PCR products. When amplified, a single amplicon of approximately 122bp was observed (as expected) for each sgRNA PCR product (dsDNA) (Figure 3.4). The Akirin-specific sgRNA PCR product had a total nucleic acid yield of 3.5µg, and the Mouse-β2m-specific sgRNA PCR product had a total yield of 4.3µg. Both sgRNA PCR products had minimal smearing and a 260/280 absorbance ratio of approximately 1.8, which was indicative of good quality DNA. When sequenced, both sgRNA PCR products had an identity of 100% to the reference sequence. These sgRNA PCR products were used as DNA templates for subsequent *in vitro* transcription.

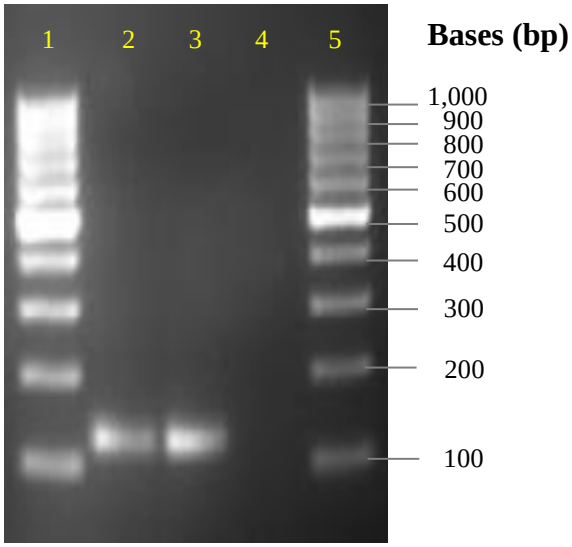


**Figure 3.4** The amplified Akirin and Mouse-β2m specific sgRNA PCR products (dsDNA), electrophoresed on a 2.5% agarose gel in TAE (100V, 70-minutes). Lane 1 and 5: O'GeneRuler™ 100bp DNA ladder, ready to use (ThermoFisher Scientific, CA92008). Lane 2: Akirin-specific sgRNA template. Lanes 3: Mouse-β2m-specific sgRNA template. Lane 4: no template control.

Once the sgRNA PCR products (dsDNA) were *in vitro* transcribed, bioanalyzer analysis was performed to assess the integrity of the sgRNA samples prior to CRISPRi inoculation. A total of four *in vitro* transcribed sgRNA replicates were synthesized for each sgRNA template. As expected, a single amplicon of 80bp was observed for the positive 18S control, while a single amplicon of approximately 107bp was observed for the Akirin and Mouse-β2m specific sgRNA. The difference in the amplicon size, from that of the PCR products, was due to the absence of the T7 promoter being included in the transcript. Since the sgRNA was created from synthetic oligonucleotide primers, RIN values were not obtained for the samples. However, from the electrophoresis, it was seen that the samples were of the correct size, with negligible degradation (Figure 3.5). The *in vitro* transcribed sgRNA was, therefore, added to the dCa9 protein to perform CRISPRi inoculation, using nano-injection.

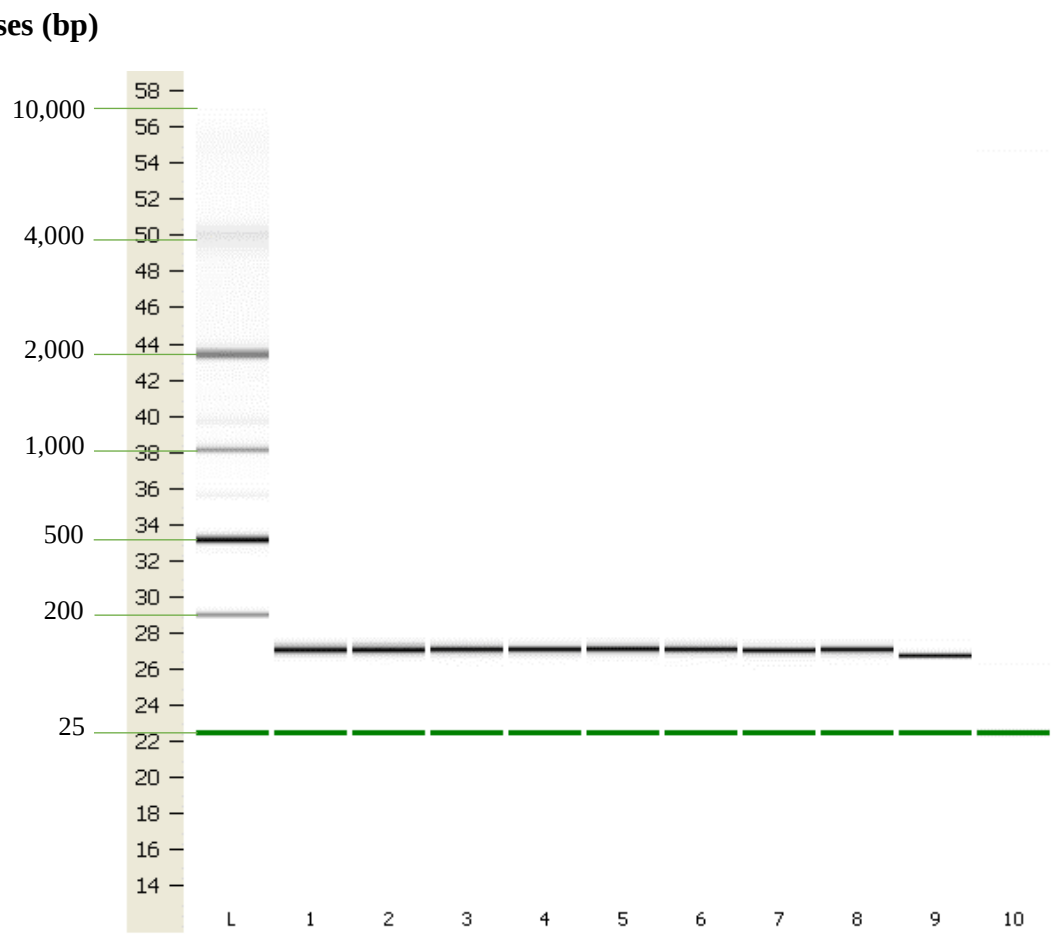


**Figure 3.5** Bioanalyzer electrophoresis displaying the integrity of the *in vitro* transcribed sgRNA samples synthesized using the MEGAshortscript™ transcription kit. L: RNA 6000 Pico ladder (Agilent, 5067-1535). Lane 1-4: Akirin-specific sgRNA. Lane 5-8: Mouse-β2m-specific sgRNA. Lane 9: *in vitro* transcription (18S) positive control. Lane 10: no template control. The green amplicons of 25bp are indicative of the Agilent RNA 6000 Pico marker.

### 3.3.2 Determination of the relative gene expression of *akirin* in *An. arabiensis* post-CRISPRi inoculation

RNA was isolated and purified from *An. arabiensis* female mosquitoes three-days post-CRISPRi inoculation. After purification, the samples had a total nucleic acid yield of approximately 140µg, and a 260/280 absorbance ratio of approximately 2.0. These results were distinctive of successful RNA isolation. RNA native gel electrophoresis was conducted to assess the integrity of the isolated RNA (Figure 3.6). A single fragment was observed at 2,000bp, which was characteristic of both the 18S and 28S rRNA fragments. This phenomenon is due to a hidden break found within insect RNA, that prevents the separation of these two fragments (Ishikawa and Newburgh, 1972; Winnebeck *et al*., 2010). A second fragment was observed at 156bp, which was distinctive of the 5.8S rRNA fragment. Minimal smearing was observed for the extracted samples, demonstrating that RNA degradation had not taken place. These characteristics were indicative of good quality RNA.

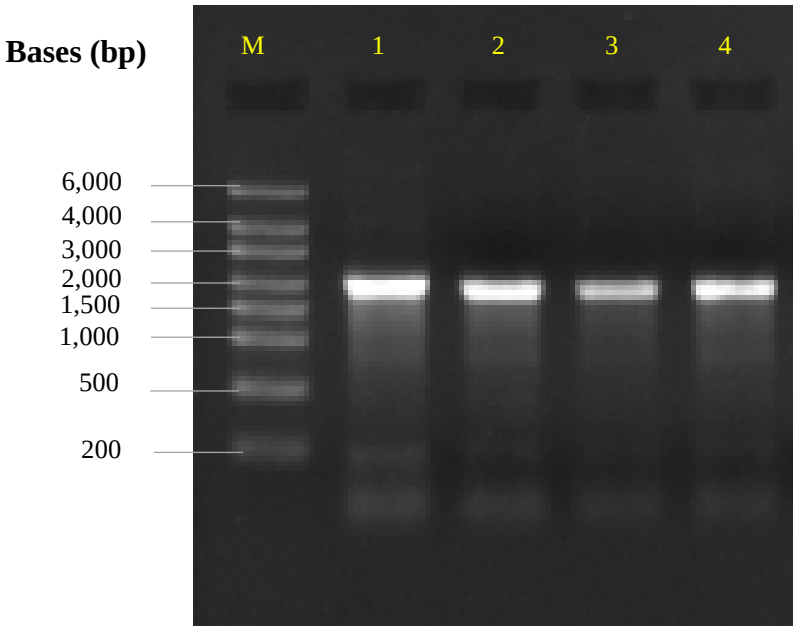


**Figure 3.6** RNA isolated from female adult *An. arabiensis* mosquitoes three-days post-CRISPRi inoculation, electrophoresed on a non-denaturing 1% agarose gel in TBE (70V, 30-minutes). M: RiboRuler™ high range RNA ladder, ready to use (ThermoFisher Scientific, SM1823). Lane 1: Akirin-specific sgRNA treated sample. Lane 2: Mouse-β2m-specific sgRNA treated sample. Lane 3: PBS treated sample. Lane 4: untreated, cold immobilised sample.

Bioanalyzer analysis was conducted to assess the RIN values of the RNA samples isolated from the female *An. arabiensis* mosquitoes three-days post-CRISPRi inoculation. All the isolated RNA samples had a RIN value of approximately 4.8 (Figure 3.7). A RIN value ranging between 3-5 is indicative of good quality insect RNA (Agilent Technologies, 2017). The first peak on the bioanalyzer electropherogram was characteristic of the pico-chip marker. This was followed by a second peak located at approximately 156bp, which was characterised as the 5.8S rRNA. The peaks indicative of the 18S and 28S rRNA were detected at approximately 2,000bp. This is due to the endogenous hidden break, which has been identified in the 28S rRNA, in most insects (Ishikawa and Newburgh, 1972; Winnebeck *et al*., 2010). The hidden break observed in insect RNA results from a double cleavage event which causes excision of the middle fragment (Winnebeck *et al*., 2010). The length of this released fragment varies between species (Agilent Technologies, 2017). The high quality of the RNA isolated from the female *An. arabiensis* mosquitoes allowed for cDNA synthesis to subsequently be performed.

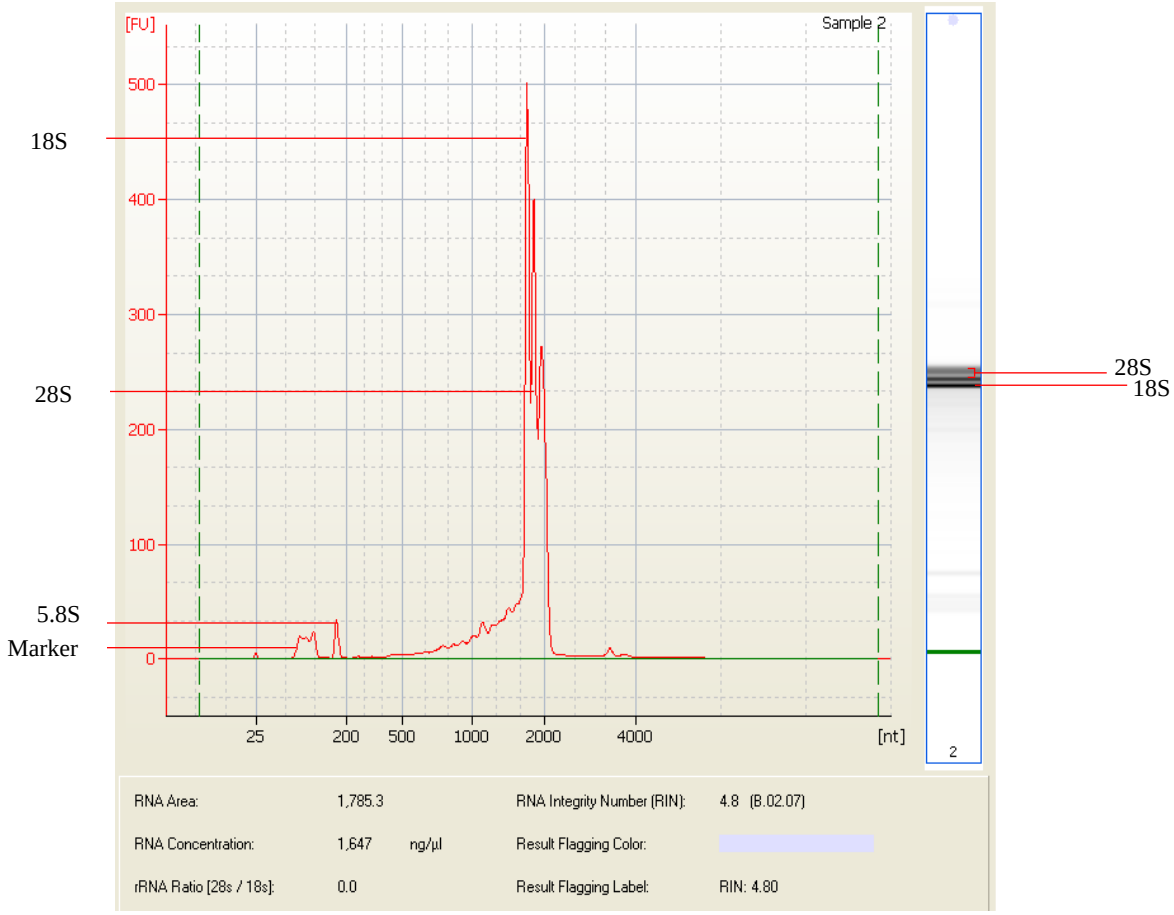


**Figure 3.7** Bioanalyzer electropherogram and electrophoresis, displaying the RIN value of the RNA samples isolated from female adult *An. arabiensis* mosquitoes three-days post-CRISPRi inoculation.

When the RNA samples were reverse-transcribed into cDNA, the cDNA samples had a mean nucleic acid yield of approximately 140µg per sample, and a 260/280 absorbance ratio of 1.8. This was indicative of good quality cDNA, which was used for RT-qPCR optimisation. An optimal primer concentration of 3.0µM, as well as an optimal annealing temperature of 62˚C, was established for both *akirin* and the reference genes. The dissociation curve was used to confirm that this annealing temperature and primer concentration did not amplify non-specific products or cause primer dimers, as the presence of these PCR products can often be distinguished by their melting characteristics (Figure 3.8). The change in fluorescence observed when dsDNA was dissociated into ssDNA attested that a single gene product was amplified for each sample.

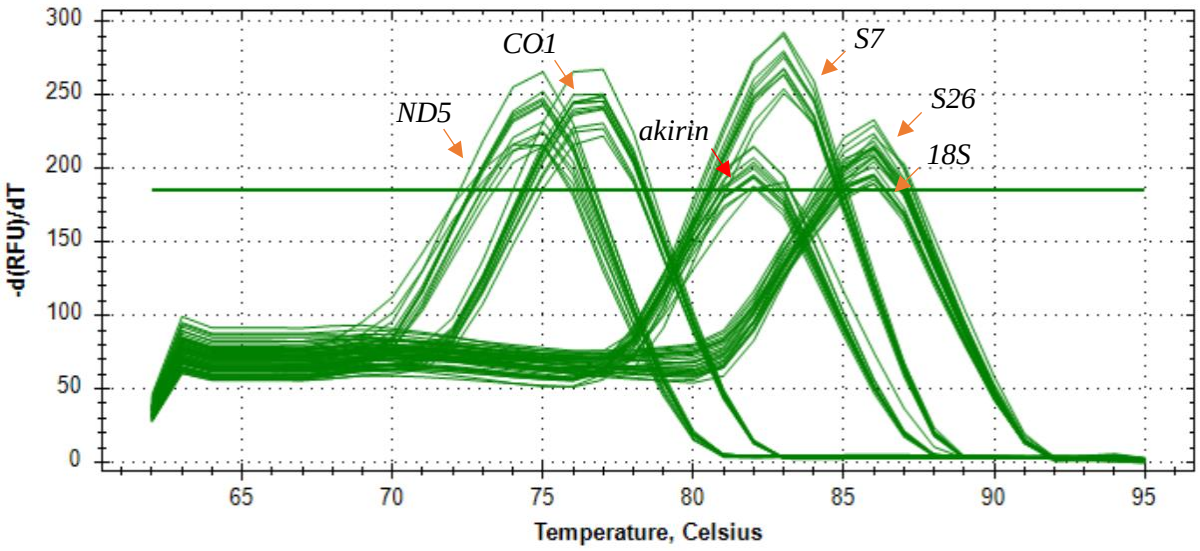


**Figure 3.8** Dissociation curve displaying the change in fluorescence observed when the dsDNA dissociated into ssDNA, upon reaching the melting temperature, for *akirin* as well as the reference genes, using an annealing temperature of 62˚C and a primer concentration of 3.0µM.

When sequenced, the PCR products that were amplified using the selected primer sets had a high percentage of sequence identity to the reference sequences (Table 3.2). This confirmed that the amplification of the primer sets was sequence-specific, and could be used to assess relative gene expression when performing RT-qPCR on the *An. arabiensis* samples.

**Table 3.2** Sequencing results obtained for both *akirin* and the reference genes, showing that the PCR products had a high percentage of sequence identity when compared to the reference sequences.

| Gene reference sequence | Primer used for sequencing | Identity to the reference sequence (bp) | Percentage identity (%) | Expected amplicon size (bp) |
|---|---|---|---|---|
| *akirin* KU973613 | Forward | (110/110) | 100 | 149 |
| | Reverse | (117/117) | 100 | 149 |
| *ND5* JX117430.1 | Forward | (84/86) | 97.5 | 121 |
| | Reverse | (92/94) | 97.9 | 121 |
| *CO1* KU187109.1 | Forward | (84/86) | 97.7 | 124 |
| | Reverse | (80/82) | 97.5 | 124 |
| *S7* AGAP010592 | Forward | (121/122) | 98.3 | 149 |
| | Reverse | (118/119) | 98.3 | 149 |
| *S26* AARA003197 | Forward | (125/126) | 99.2 | 160 |
| | Reverse | (125/126) | 99.2 | 160 |
| *18S* AF417777.1 | Forward | (97/97) | 100 | 130 |
| | Reverse | (102/102) | 100 | 130 |

The *S7* and *S26* reference genes were selected to normalise the relative expression of *akirin*, based on the stability value obtained when NormFinder was used to assess the variation in gene expression between the replicates of the various treatments. The stability value was determined by analysing the Cq values generated when each sample was amplified, and was subsequently expressed as a numerical value. A value near 0 indicated that there was minimal variation between the various treatments, while a value near 1 indicated a high degree of variation between the treatments. A stability value of 0.032 was obtained for *18S*, 0.031 for *CO1*, and 0.030 for *ND5*, while the *S7* reference gene had a stability value of 0.018, and the *S26* reference gene had a stability value of 0.011. The latter reference genes were used to normalise the relative gene expression of *akirin* expression once RT-qPCR had been performed.

Once normalised, the relative expression software tool (REST©) was used to determine the change in relative *akirin* expression amongst the various treatments. Samples treated with the Akirin-specific sgRNA:dCas9 complex showed a significant reduction in *akirin* expression, by a mean factor of 0.322 (S.E. range 0.16-0.54) ($p$<0.01), when compared to the control samples treated with the Mouse-β2m-specific sgRNA:dCas9 complex. This was a 67.8% decrease in *akirin* expression. As expected, no significant change was detected when the relative gene expression of *akirin* was assessed between the control samples (Figure 3.9). This attested that *akirin* expression could be suppressed in *An. arabiensis* by performing CRISPRi. It should, however, be noted that additional research would need to be conducted to determine how long the effects of *akirin* knockdown would persist for, when using the dCas9 system.

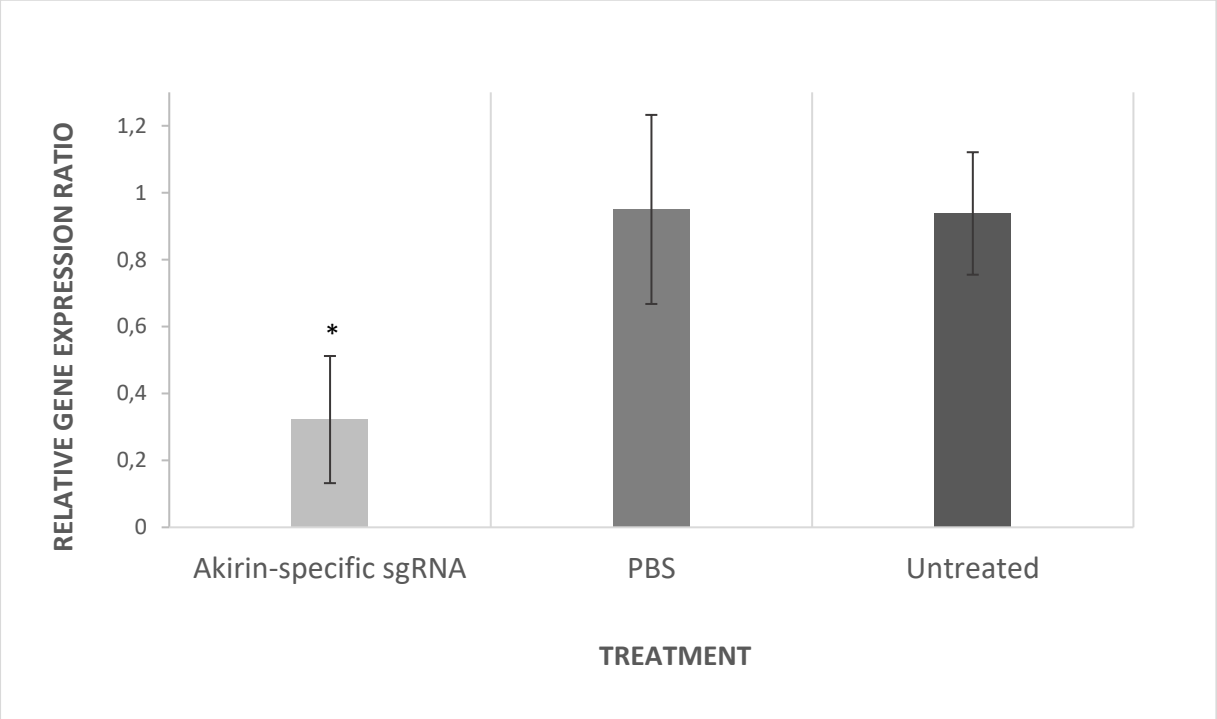


**Figure 3.9** The change in relative *akirin* expression assessed three-days post-CRISPRi treatment. The relative expression of *akirin* was significantly downregulated by a mean factor of 0.32 (S.E. range 0.16-0.54) ($p$<0.01) when comparing the expression ratios of *akirin* between the samples treated with Akirin-specific sgRNA and the samples treated with Mouse-β2m-specific sgRNA (negative control). The relative expression of *akirin* remained unchanged when comparing the expression ratios of the negative control samples to the PBS treated samples ($p$=0.91) and the untreated, cold immobilised samples ($p$=0.56). The asterisk shows a significant change in the relative expression of *akirin* in comparison to the negative control treatment, while the error bars on the graph are indicative of the S.E. between the treatment replicates.

### 3.3.3 Evaluation of the effect of *akirin* suppression, by CRISPRi, on *An. arabiensis* longevity

The overall vector longevity was significantly reduced in the *akirin* knockdown female *An. arabiensis* mosquitoes when compared to the three control treatments (Figure 3.10). *Anopheles arabiensis* which were treated with Akirin-specific sgRNA had an overall mortality of 18-days post-inoculation, while the *An. arabiensis* that were treated with Mouse-β2m-specific sgRNA had an overall mortality of 26-days post-inoculation. The *An. arabiensis* treated with PBS treated and the untreated mosquitoes had an overall mortality of 27-days. This was a 33% decrease in vector survival when longevity was compared between the *akirin* knockdown samples and the control treatments (Log-rank: $\chi^2$=20.05, $p$<0.01, DF=3). When assessing the mean longevity, the *An. arabiensis* treated with Akirin-specific sgRNA had a mean survival time of 13-days post-inoculation, the *An. arabiensis* that were treated with Mouse-β2m-specific sgRNA had a mean survival time of 16-days post-inoculation, the PBS treated *An. arabiensis* had a mean survival time of 17-days post-inoculation, and the untreated samples had a mean survival time of 16-days. This was an 18.7% decrease in vector longevity, when the mean survival time was compared between the *akirin* knockdown samples and the control treatments (Log-rank: $\chi^2$=9.45, $p$=0.02, DF=3).

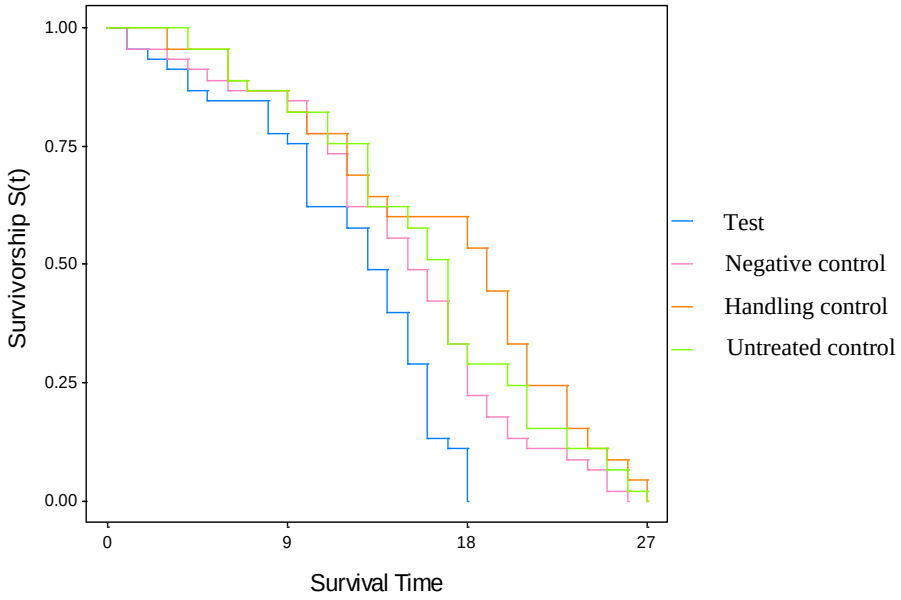


**Figure 3.10** Kaplan-Meier survival analysis of *An. arabiensis* assessed post-CRISPRi treatment. Samples treated with Akirin-specific sgRNA (blue) reached 100% mortality 8-days before the samples treated with Mouse-β2m-specific sgRNA (pink), 9-days before the samples treated with PBS (orange), and 9-days before the untreated samples (green). This conveyed a 33% decrease in vector longevity in relation to the control treatments (Log-rank: $\chi^2$=20.05, $p$<0.01, DF=3).

### 3.4 Discussion

The flexible nature of the RNA-guided DNA recognition tool, CRISPRi, allows for the transient control of gene expression without altering the genomic sequence (Larson *et al*., 2013). The downregulation of gene expression, using the nuclease-deficient Cas9 protein, causes a partial loss of gene function. This gene regulatory technique is imperative for understanding the function of essential genes, such as those involved in embryonic development or innate immunity. The CRISPRi technique was used to assess the role of Akirin in *An. arabiensis*. Since this was still a relatively novel technique, CRISPRi had not yet been performed in Culicidae using the dCas9 protein. This platform was therefore also used to determine if the CRISPRi-dCas9 system could be used to suppress the transcription elongation of Akirin in *An. arabiensis*, and whether a change in the expression of *akirin* would affect vector longevity.

The Akirin-specific sgRNA was designed and synthesised to target the non-template strand of the Akirin DNA sequence in *An. arabiensis.* When the Akirin-specific sgRNA was administered to *An. arabiensis*, along with the dCas9 protein, a physical barrier was caused to the passage of RNA polymerase II when Akirin was being transcribed (Qi *et al.*, 2013). During transcriptional elongation, RNA polymerase II unwinds the double-stranded DNA, and uses the template strand to create mRNA (Larson *et al*., 2013). The physical barrier, caused by the dCas9 protein, repressed the transcription elongation of Akirin, which caused a 67.8% decrease of relative *akirin* expression in *An. arabiensis*, when compared to the control treatments. This decrease in *akirin* expression was 43.8% more than the decrease in *akirin* expression obtained when Akirin was targeted using Akirin-specific dsRNA, when performing RNAi (Letinić *et al*., 2020).

The effects observed when performing RNAi is mainly determined by the distribution of the RNAi signal within the organism (Sijen *et al.,* 2001). This amplified RNAi systemic response is largely due to the presence of RNA-dependent RNA polymerase (RdRP) (Sijen *et al.,* 2001). However, many insects, including mosquitoes, do not possess RdRPs or RdRPs homologues in their genomes (Obbard *et al*., 2009). Since CRISPRi is mediated by a dCas9 protein that limits the transcriptional activity of RNA polymerase, this system does not need any additional transcription inhibitory components. This demonstrates the promising alternative that the

CRISPRi-dCas9 system offers for organisms that do not respond to traditional gene suppression techniques, such as RNAi.

The 67.8% decrease of relative *akirin* expression, due to the CRISPRi treatment, resulted in an 18% significant decrease in *An. arabiensis* vector longevity when analysing the mean survival time. The effect of *akirin* knockdown on vector fecundity and fertility were not assessed, using CRISPRi, due to challenges with obtaining additional reagents and the financial constrains prevented the ordering of additional kits. The decrease in vector longevity, however, was consistent with previous Akirin gene regulation studies conducted on *An. coluzzii,* where the downregulation of *akirin* expression resulted in a 14% significant decrease in vector survival when performing RNAi (da Costa *et al*., 2014). A possible reason for this could be that *akirin* knockdown impairs the gene expression of several AMPs, which may disrupt bacterial homeostasis in the mosquitoes midgut (Mukherji *et al.,* 2013; Debalke *et al.,* 2019). This weakens the innate immune defence, causing enhanced sensitivity to Gram-negative bacterial infection, which subsequently affects vector survival (Ferrandon *et al.,* 2007; Goto *et al.,* 2008; Nowak *et al.,* 2012; Bonnay *et al.,* 2014).

As aforementioned, mosquito longevity is a critical factor affecting malaria transmission. The female mosquito needs to survive longer than 14-days post-gametocyte infection, to transmit the *Plasmodium* parasite (Bray and Garnham, 1982). *Anopheles arabiensis* which were treated with Akirin-specific sgRNA had a mean survival time of 13-days post-inoculation. This decrease in vector longevity was similar to that of the *An. arabiensis* treated with Akirin-specific dsRNA, which had a mean survival time of 15-days post-inoculation (Letinić *et al*., 2020). Reducing half of the female vector population, prior to the completion of the extrinsic incubation period, would ultimately have a substantial impact on malaria transmission.

Although the results from this study are similar to those of previous Akirin gene regulation studies that have been performed on Culicidae, the techniques used in these studies targeted different levels of gene regulation. CRISPRi binds to DNA to regulate transcription, while RNAi binds to mRNA to regulate translation (Larson *et al*., 2013; Qi *et al.,* 2013). Therefore, it becomes problematic when directly comparing the outcomes of these two techniques. A novel type II system CRISPR nuclease, called Cas13, has recently been discovered, and repurposed to directly target mRNA without the requirement of a PAM sequence (Abudayyeh *et al.,* 2016; Abudayyeh *et al.,* 2017; Freije *et al.,* 2019). This system has proven highly

efficient at regulating gene expression in *An. gambiae* and *Ae. aegypti* (Kulkarni *et al.*, 2020), and could therefore be used to allow for a more accurate comparison between the two gene-editing techniques. Comparing inter-study data does, however, have its limitations. This can be demonstrated when comparing the results of the mosquito lifetable-parameters, such as vector longevity, of the untreated control treatments between the various techniques analysed in this thesis. Additionally, the effects of the CRISPRi-Cas13 system have not been evaluated on genes that are expressed in the midgut, salivary glands, and ovaries (Kulkarni *et al.*, 2020). This may be problematic if used to target Akirin, as previous Akirin gene regulation analysis performed in *An. coluzzii* showed that *akirin* expression was downregulated by 16-40% in the mosquito midgut, and 25-65% in the remaining tissue (da Costa *et al*., 2014). Subsequently, further studies would need to be conducted to evaluate the use of the CRISPRi-Cas13 system, on targets such as Akirin, in *An. arabiensis*.

Currently, CRISPRi has only been used to determine the regulatory function of certain genes however, this technology could be further developed to potentially target wild *An. arabiensis* vector populations. To achieve a similar detrimental phenotype in wild populations, CRISPRi triggers must be delivered to *An. arabiensis* with consideration for environmental and abiotic factors, using field feasible applications. An *E. coli* expression system could be used to express plasmids containing the sgRNA:dCas9 construct, which could subsequently be delivered to *An. arabiensis* by oral ingestion (Qualls *et al.*, 2014; Zhu *et al.*, 2015; Ding *et al.*, 2016). This formulation could be administered to the wild populations of *An. arabiensis* using attractive-toxic sugar bait stations near breeding sites (Qualls *et al.*, 2014; Zhu *et al.*, 2015). However, additional research would need to be performed to evaluate the stability and feasibility of such a system, as well as the effect of *akirin* knockdown on other mosquito life-table parameters, such as ovipositioning, when using CRISPRi. Furthermore, whilst extending beyond the scope of the current study, this system could be compared with an active Cas9 system.

This study, therefore, shows that the CRISPRi-dCas9 system can be used to repress transcription elongation in *An. arabiensis.* It also shows that reducing *akirin* expression in *An. arabiensis* can cause a significant decrease in vector longevity. Targeting Akirin, in *An. arabiensis,* could reduce the mean survival time of the vector population, which would aid in the reduction of malaria transmission. Currently, CRISPRi has only been used to determine the regulatory function of genes such as *akirin* however, this technology could be further developed, to aid as an additional tool for *An. arabiensis* vector population control.

# CHAPTER 4: RNA INTERFERENCE

**Objective:** Determine the effect of *akirin* knockdown on *An. arabiensis* lifetable parameters, using RNA mediated interference.

The work described in Chapter 4 was published on 12 February 2020 (Appendix E): **Letinić, B.D., Dahan Moss, Y. and Koekemoer, L.L.**, 2020. Characterising the effect of *akirin* knockdown on *Anopheles arabiensis* (Diptera: Culicidae) reproduction and survival, using RNA-mediated interference. *PloS One*, *15*(2), p.e0228576.

## 4.1 Introduction

RNA interference (RNAi) is a naturally occurring defence mechanism that was first observed in plants and fungi (Napoli *et al.*, 1990; Romano and Macino, 1992). This gene regulation mechanism is activated upon aberrant transcription or viral infection. RNA interference has since been adapted as a gene-editing tool, which is capable of repressing gene expression post-translation. The capacity of this gene-editing tool was first demonstrated when the expression of the *unc-22* gene was manipulated in *Caenorhabditis elegans* (Fire *et al.*, 1998).

Diminished *unc-22* expression is known to cause a severe twitching phenotype in *C. elegans*, as the *unc-22* gene encodes for an abundant, yet nonessential, myofilament protein (Fire *et al.*, 1998). When a mixture of sense and antisense RNA was administered into the *C. elegans*, by nano-injection, severe twitching resulted. This change in phenotype inferred repression of *unc-22* gene expression, which demonstrated the ability of using gene-specific dsRNA to repress gene expression post-transcription (Fire *et al.*, 1998).

Most eukaryotic organisms, including insects, have similar mechanisms that allow for sequence-specific post-transcriptional gene silencing to take place when dsRNA is present. This highly specific defence reaction results in the degradation of the targeted mRNA due to the formation of small RNAs (Fire *et al.*, 1998). Several small RNAs have been discovered in both insects and other multicellular organisms. These small RNAs include piwi-interacting RNAs (piRNAs), microRNAs (miRNAs), and short interfering RNAs (siRNAs) (Siomi and Siomi, 2009; Brodersen and Voinnet, 2009).

During RNAi, long endogenous or exogenous dsRNA that has been expressed in or introduced into a cell, is subsequently processed into small RNA duplexes by the ribonuclease III (RNase III) enzyme, Dicer (Zamore, 2001; Meister and Tuschl, 2004). These small RNA duplexes are made up of two strands, namely the guide strand, and the passenger strand (Hutvágner and Zamore, 2002). When unwound, the guide strand forms a bond with the Argonaut-2 (Ago2) protein, to create the RNA-induced silencing complex (RISC), while the passenger strand is degraded (Hutvágner and Zamore, 2002). The guide strand directs the RISC complex to the target messenger RNA (mRNA) by complementary base pairing (Hutvágner and Zamore, 2002). The RISC-bound endonuclease activity of Ago2 causes the cleavage of target mRNA (Figure 4.1) (Zamore, 2001). The cleaved mRNA is subsequently degraded, which causes translation inhibition (Meister and Tuschl, 2004).

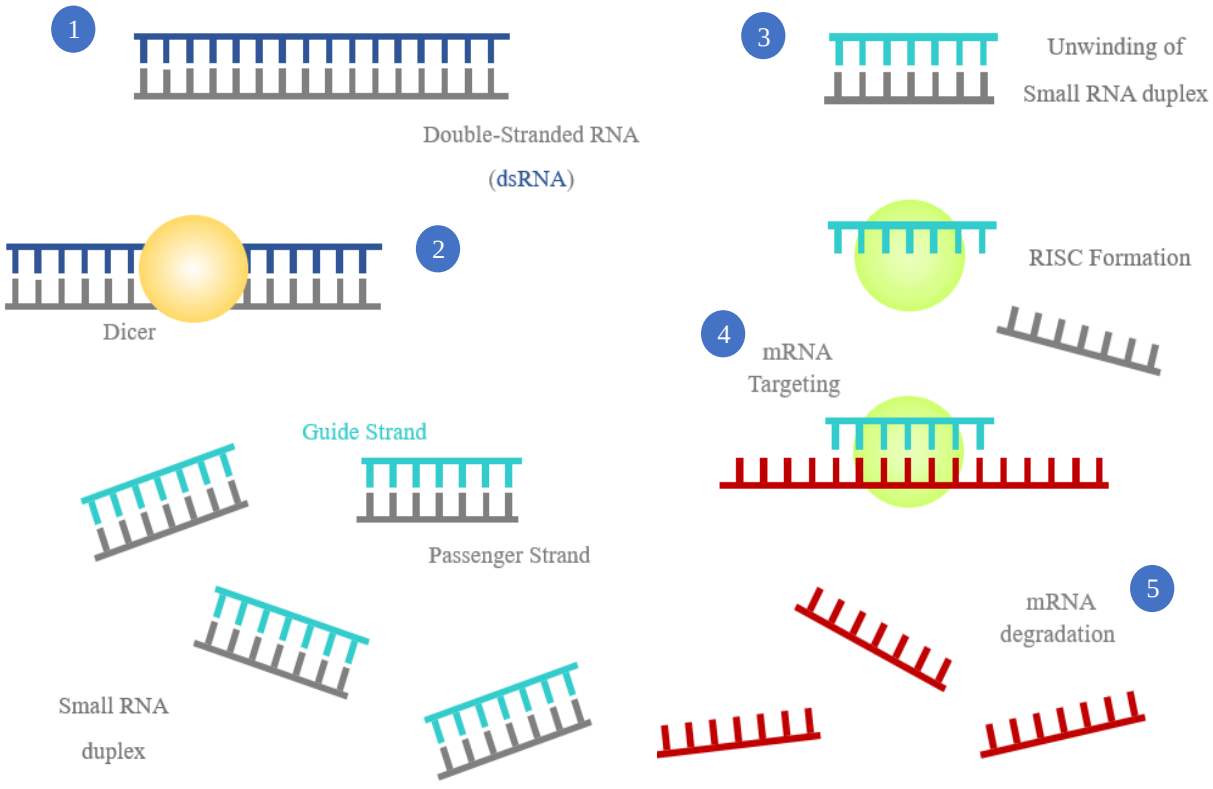


**Figure 4.1** An overview of the RNAi pathway. 1: RNA interference is initiated upon the expression or introduction of long endogenous or exogenous dsRNA (dark blue) into a cell. 2: The RNase III enzyme, Dicer (yellow), cleaves the dsRNA into small RNA duplexes (light blue). 3: The guide strand of the small RNA duplex forms a bond with the Argonaut protein, Silencer, to create the RNA-induced silencing complex (RISC) (green). 4: The guide strand directs and binds the RISC complex to the target mRNA (red), causing cleavage of the mRNA transcript. 5: The cleaved mRNA is subsequently degraded, inhibiting further translation.

The effects observed when performing RNAi is largely determined by the distribution of the RNAi signal within the organism. Although the core mechanisms of the RNAi technique are conserved throughout eukaryotes, RNAi signal distribution differs between species (Saleh *et al.,* 2006). In some organisms, the RNAi signal can spread throughout the organism's body (Tomoyasu *et al.,* 2008). This is referred to as a systemic RNAi signal, and elicits a strong RNAi response. In other organisms, dsRNA uptake causes localised gene silencing without a systemic distribution of the RNAi signal (Roignant *et al.,* 2003). Therefore, the RNAi signal does not spread throughout the entire body of the organism (Saleh *et al.,* 2006).

A strong RNAi systemic response is observed when performing RNAi in *C. elegans*. This amplified RNAi systemic response is largely due to the presence of RNA-dependent RNA polymerase (RdRP) (Sijen *et al.,* 2001). Homologues of RdRPs are not present in mosquito genomes, however, they have been identified in genomes of other arthropods such as ticks (Obbard *et al*., 2009). This mechanism is most likely accountable for the robust response of dsRNA-mediated RNAi observed in various tick species (Almazán *et al.,* 2005; Almazán *et al.,* 2010). Another mechanism that has been found to enable the uptake of long dsRNA in *C. elegans* is the RNA channel transporter gene called *SID-1* (systemic RNA interference deficiency-1) (Winston *et al*., 2002). However, *Dipterans* lack SID homologues (Saleh *et al.,* 2006; Kurscheid *et al.,* 2009).

Despite the lack of RdRP and SID proteins, there may be an alternative dsRNA uptake mechanism that allows for a systemic RNAi response in Culicidae (Zhu *et al.,* 2003; Boisson *et al.,* 2006). Although this mechanism has not yet been defined, successful RNAi gene manipulation studies have been performed in various species of Culicidae, through soaking, feeding, and nano-injection (Blandin *et al.,* 2002; Whyard *et al.,* 2009; Lopez-Martinez *et al.,* 2012). Effective RNAi experiments have been performed in all the developmental stages of mosquitoes however, the majority of these studies were performed on the adult life stage, using nano-injection.

The first successful RNAi trial conducted on adult *An. gambiae* mosquitoes, by nano-injection, effectively repressed the gene expression of the antimicrobial peptide *defensin* (Blandin *et al.,* 2002). A video protocol describing the detailed method for nano-injection of dsRNA into adult *Anopheles* mosquitoes was subsequently produced (Garver and Dimopoulos, 2007). This set

the premise for other RNAi gene manipulation studies performed in *Anopheles* using nano-injection (Pondeville *et al.*, 2013), including the repression of *akirin* expression in *An. coluzzii* (da Costa *et al.*, 2014). Nano-injection was, therefore, used to repress *akirin* expression, in *An. arabiensis*, by performing RNAi (Letinić *et al*., 2020).

Since short dsRNA have had the most success with RNAi (Siomi and Siomi, 2009), *akirin* knockdown was performed using Akirin-specific siRNA as well as Akirin-specific long dsRNA. Both RNA-interference inducing molecules were administered to *An. arabiensis*, to target Akirin mRNA. The change in *akirin* expression was assessed by performing RT-qPCR. After this, the phenotypic effect caused by the change in *akirin* expression was assessed in adult female *An. arabiensis*. Mouse-β2m was once again used as a negative control, since this protein does not have a complementary sequence within the *An. arabiensis* genome (da Costa *et al.*, 2014). This allowed for the characterisation of the biological function of Akirin, in *An. arabiensis*, with regards to mosquito lifetable parameters.

*Anopheles arabiensis* survival was assessed to determine whether *akirin* knockdown, by RNAi, affected vector longevity. Akirin is a vital component of various biological pathways including the IMD signalling pathway, which is needed for defence against bacterial infection (Ferrandon *et al.*, 2007; Goto *et al.*, 2008). Consequently, repressing *akirin* expression would have an impact on vector survival, which is a crucial factor in malaria transmission. The change in *akirin* expression was also assessed on vector fecundity and vector fertility, since Akirin is also functionally important for reproduction (da Costa *et al.*, 2014; Almazán *et al.*, 2005; Almazán *et al.*, 2010).

Since the biological function of Akirin had not yet been investigated in *An. arabiensis*, these results were used to determine whether targeting Akirin could be further developed as an additional tool for *An. arabiensis* vector control. Reducing *An. arabiensis* survivorship as well as reproductive fitness would ultimately cause a reduction in vector population abundance. A reduction in vector abundance would aid in the reduction of malaria transmission.

## 4.2 Materials and Methods

### 4.2.1 *In vitro* transcription of Akirin-specific dsRNA to target Akirin mRNA in *An. arabiensis,* when performing RNAi by nano-injection

Two separate techniques were used to repress the expression of endogenous *akirin* mRNA in *An. arabiensis* (Letinić *et al*., 2020). The first technique used commercially available small interfering RNAs (siRNAs) that were synthesised by ThermoFisher Scientific, using Ambion® *in vivo* chemical modifications, to ensure minimal off-target activity. Putative siRNAs were selected based on the *Silencer*® Select algorithm, and were used to analyse the full coding sequence of each gene. RNAi-mediated knockdown, using Ambion® siRNA, was optimised using GapDH-specific siRNA as a positive control (ThermoFisher Scientific, s541529). *GapDH* was significantly downregulated by a mean factor of 0.16 (S.E. range 0.11-0.28) ($p$=0.03) when conducting RT-qPCR (Letinić *et al*., 2020). Ambion® *in vivo* custom-designed siRNA was used to target Akirin (ThermoFisher Scientific, s541552), while the Ambion® pre-designed Mouse-β2m-specific siRNA (*in vivo)* was used as a negative control (ThermoFisher Scientific, s62844). See Appendix B for siRNA sequences.

The second technique used *in vitro* transcribed long dsRNA, which was synthesised using RNA that was isolated from 1-day-old female *An. arabiensis,* using TRI reagent (Sigma-Aldrich, T9424), and was quantified using a NanoDrop™ spectrophotometer. The integrity of the isolated RNA was assessed using a 1% non-denaturing agarose gel in TBE, which was electrophoresed for 30-minutes at 70V (Hummon *et al.*, 2007). The isolated RNA was reverse-transcribed into cDNA using the QuantiTect® reverse-transcription kit (Qiagen, 205310). See chapter 2 for detailed methods. The cDNA was amplified using the Akirin-specific dsRNA forward primer ($^{5'}$GGTACTTTGGCAGTCGTTGTAGTTGC$^{3'}$), and the Akirin-specific dsRNA reverse primer ($^{5'}$GGTACTCACCTGCTTGAAGGTGAACA$^{3'}$), which contained T7 promoters ($^{5'}$TAATACGACTCACTATAG$^{3'}$), that were appended at the 5′ end of the sequences. The T7 promoters allowed for *in vitro* transcription to take place following PCR.

The PCR was performed using cDNA (250ng), 1x PCR buffer (10mM Tris-HCl, 5mM KCl), 1mM dNTP's, 3mM $MgCl_2$, 0.4µM forward primer, 0.4µM reverse primer, and 0.1U *Taq* polymerase, which was made up to a final volume of 25µL using nuclease-free water. The PCR was conducted by heating the sample to 94˚C for 5-minutes, followed by 40 PCR cycles (94˚C

for 30-seconds, 52˚C for 1-minute, 72˚C for 1-minute), and a final extension at 72˚C for 5-minutes. The PCR products were purified using the QIAquick® PCR purification kit (Qiagen, 28104), as per the manufacturer's specifications. The purified PCR products were quantified using the NanoDrop™ spectrophotometer, after which the integrity and size of the purified PCR products (250ng) were assessed using a 1% agarose gel in TAE, which was electrophoresed for 75-minutes at 110V, prior to *in vitro* transcription.

The MEGAscript® RNAi kit (Invitrogen, AM1626) was used to assemble the transcription reaction, by generating two complementary RNA transcripts. In a 20µL reaction, 1-fold of ATP, CTP GTP, UTP, T7 enzyme mix, and reaction buffer, were added to 1µg of dsDNA template. The sample reactions were incubated at 37˚C for 4-hours. Nuclease digestion was performed to digest residual template DNA and ssRNA. Nuclease digestion was conducted by adding 2.5x digestion buffer, 2µL DNaseI, 2µL RNase, and 21µL nuclease-free water to the *in vitro* transcribed dsRNA product. This reaction was incubated for 1-hour at 37˚C, to allow for the template DNA to digest. The digested DNA, free nucleic acids, and residual proteins were removed from the dsRNA using the filter cartridge provided in the MEGAscript® RNAi kit, as specified by the manufacturer. The eluted purified *in vitro* transcribed dsRNA was quantified using the NanoDrop™ spectrophotometer, and the integrity was assessed using a 1% agarose gel in TAE, which was electrophoresed for 75-minutes at 110V.

The Nanoinject II (Drummond, 3-000204) was used to conduct RNAi inoculation on 1-day-old female *An. arabiensis* mosquitoes (Letinić *et al*., 2018). Cold anesthetised mosquitoes were inoculated with 69nL (3mg/mL) of Akirin-specific dsRNA, Akirin-specific siRNA, or Mouse-β2m-specific siRNA, via the thorax. A subset of cold anesthetised mosquitoes, that were injected with PBS, was used as a handling control, while a subset of mosquitoes that were not subjected to inoculation but had undergone cold anesthetisation, was used as an untreated control. Inoculations were conducted using a total of 300 female mosquitoes per treatment. Fifty mosquitoes were randomly selected from each treatment for RT-qPCR analysis (10 mosquitoes per replicate, 5 replicates), while 150 female mosquitoes were randomly selected for longevity analysis (30 mosquitoes per replicate, 5 replicates). The remaining 100 female mosquitoes from each treatment were used to analyse vector fertility and fecundity (20 mosquitoes per replicate, 5 replicates). All treatments were carried out in 32.5cm$^3$ insect rearing cages (BugDorm®, 211476) (Letinić *et al*., 2020).

### 4.2.2 Determination of the relative gene expression of *akirin* in *An. arabiensis* post-RNAi inoculation

RNA was extracted from whole mosquito samples, three-days post-RNAi inoculation, using TRI reagent (Sigma-Aldrich, T9424). The isolated RNA was reverse-transcribed into cDNA using the QuantiTect® reverse-transcription kit. Quantitative-PCR was conducted using 1x iQ™ SYBR® Green supermix (Bio-Rad, 170-8887), as specified in chapter 3.

### 4.2.3 Evaluation of the effect of *akirin* suppression, by RNAi, on *An. arabiensis* lifetable parameters

Mosquito fecundity and fertility were analysed post-inoculation, using a total of 100 female mosquitoes per treatment (20 mosquitoes per replicate, 5 replicates). The female mosquitoes were placed in cages with untreated 2-day-old male mosquitoes (20 mosquitoes per cage) for a period of four days, to allow mating to take place, after which the male mosquitoes were removed from the cages (Letinić *et al*., 2020). Two subsequent blood meals were offered to mated females over five days (Munhenga *et al.*, 2016). After receiving the second blood meal, each female was transferred into a separate paper cup (8cm diameter, 9cm height). A net was fastened over the top of each cup using an elastic band. Approximately 20mL of distilled water was poured into each cup. Mosquitoes were maintained on a 10% sucrose solution until oviposition had taken place. Vector fecundity was determined by calculating the mean number of eggs laid per female. The hatch rate was monitored over ten days. Eggs that did not hatch ten days post-oviposition were deemed infertile. Vector fertility was determined by calculating the mean percentage of hatchlings per female (Letinić *et al*., 2020). Both fecundity and fertility were compared between the treatment and controls, using a one-way analysis of variance (ANOVA), and a Tukey HSD post-hoc test. This was done to analyse the differences in means of each variable (Munhenga *et al.*, 2016), and report on correlations that were observed.

Longevity was assessed to determine whether *akirin* knockdown affected *An. arabiensis* survival. Longevity was evaluated using a total of 150 female mosquitoes per treatment (30 mosquitoes per replicate, 5 replicates). The rate of survival was monitored until 100% mortality was reached for all five treatments (Akirin-specific dsRNA, Akirin-specific siRNA, Mouse-β2m-specific siRNA, PBS, and untreated). See chapter 2, section 7, for the detailed method.

## 4.3 Results

### 4.3.1 *In vitro* transcription of Akirin-specific dsRNA to target Akirin mRNA in *An. arabiensis,* when performing RNAi by nano-injection

RNA was successfully extracted from *An. arabiensis* mosquitoes, to synthesize dsRNA. When quantified, the RNA had a 260/280 absorbance ratio of approximately 2.0, which was indicative of pure RNA. The integrity of the extracted RNA was assessed by performing a non-denaturing agarose gel electrophoresis (Figure 4.2). The fragment observed at 2,000bp (18S and 28S amplicon), as well as the less bright fragment observed at 156bp (5.8S rRNA), was characteristic of insect RNA (Ishikawa and Newburgh, 1972; Winnebeck *et al*., 2010). Minimal degradation of the extracted samples was observed, which was indicative of good quality insect RNA. The RNA was subsequently reverse-transcribed into cDNA.

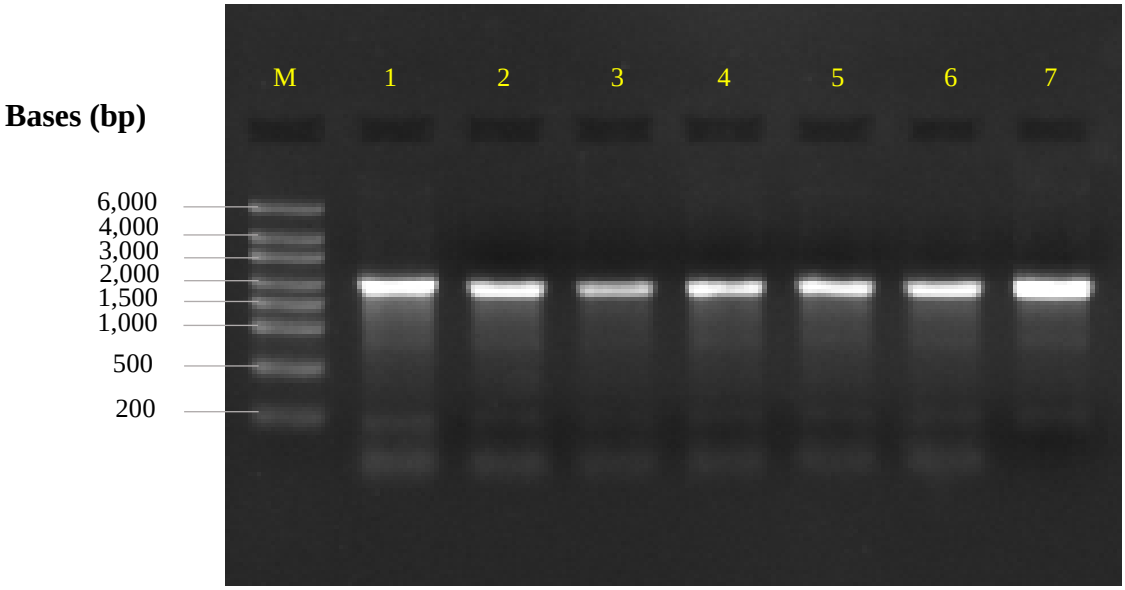


**Figure 4.2** RNA isolated from female adult *An. arabiensis* mosquitoes, electrophoresed on a non-denaturing 1% agarose gel in TBE (70V, 30-minutes). M: RiboRuler™ high range RNA ladder (ThermoFisher Scientific, SM1823). Lanes 1 to 7: RNA extracted from 1-day-old untreated female adult *An. arabiensis* mosquitoes.

The RNA isolated from *An. arabiensis* was reverse-transcribed into cDNA. After purification, the cDNA samples had a mean nucleic acid yield of approximately 10µg, and a 260/280 absorbance ratio of 1.8. This was indicative of pure DNA. The cDNA was amplified using the Akirin-specific dsRNA primers designed by da Costa *et al.* (2014). The integrity and specificity of the Akirin PCR products was assessed using a 1% agarose gel in TAE. When amplified, a single amplicon at approximately 557bp was observed (as expected) for each *An. arabiensis* Akirin PCR product (Figure 4.3) When quantified, the amplified products had a mean nucleic acid yield of approximately 13µg of DNA, and a 260/280 absorbance ratio of 1.8, which was indicative of good quality DNA. When sequenced, the amplified *An. arabiensis* Akirin PCR products had an identity of 99% to the reference sequence. These Akirin PCR products were subsequently *in vitro* transcribed into dsRNA.

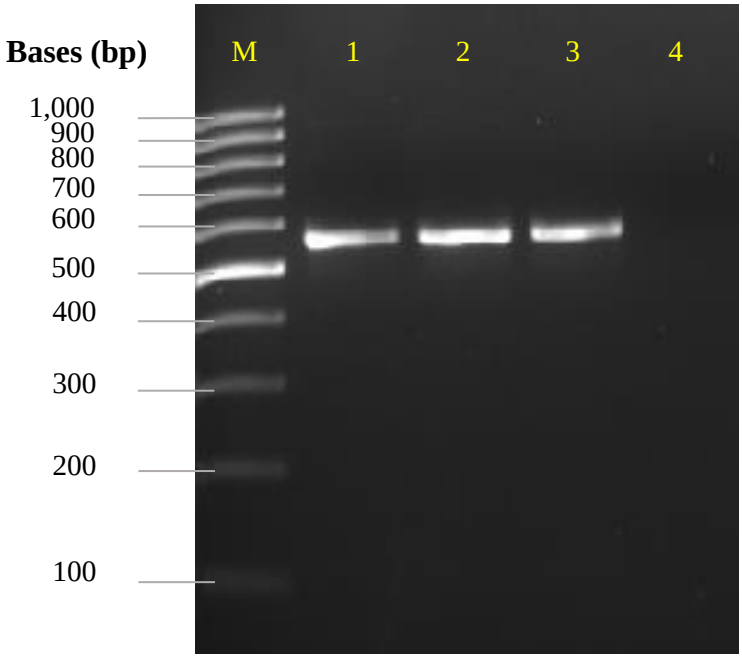


**Figure 4.3** PCR products synthesized from cDNA, that was reverse-transcribed from RNA isolated from 1-day-old female adult *An. arabiensis* mosquitoes, electrophoresed on a 1% agarose gel in TAE (110V, 75-minutes). M: O'GeneRuler™ 100bp DNA ladder, ready to use (ThermoFisher Scientific, SM1143). Lane 1-3: Akirin PCR products. Lane 4: no template control.

The Akirin-specific PCR products were *in vitro* transcribed into dsRNA to perform RNAi in *An. arabiensis*. Quantification was conducted on the *in vitro* transcribed dsRNA once nuclease digestion and purification had been performed. The *in vitro* transcribed dsRNA samples had a mean nucleic acid yield of approximately 50µg, and a 260/280 absorbance ratio of approximately 2.00, which was characteristic of good quality RNA. The dsRNA products were analysed using a 1% agarose gel in TAE (Figure 4.4), to confirm that the correct product was transcribed. A single fragment of 557bp (Lane 1-3) was observed for each *in vitro* transcribed product, which was indicative of the expected Akirin-specific dsRNA product. The low density of the agarose gel caused the dsRNA fragment to move slower than the dsDNA ladder. This phenomenon was due to molecular stiffness differences between the two molecules (Livshits *et al.*, 1990).

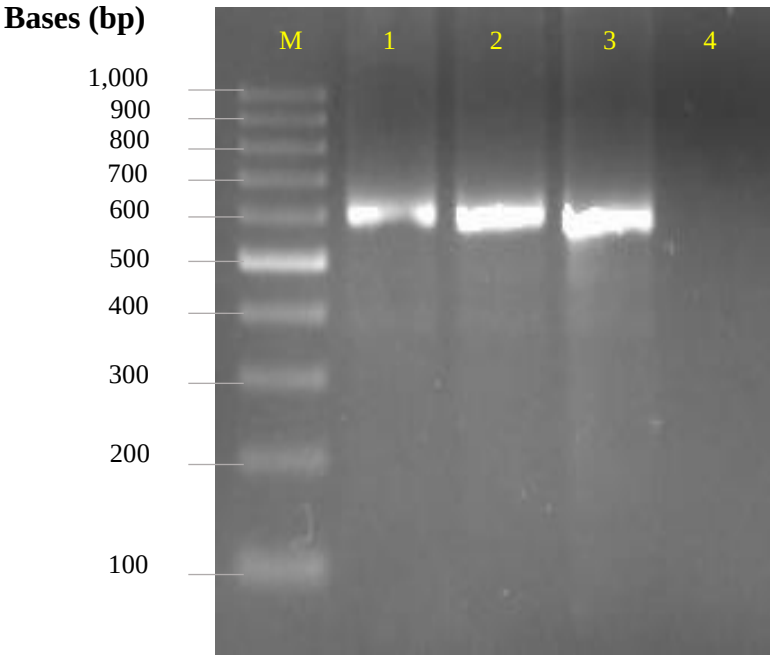


**Figure 4.4** *In vitro* transcribed dsRNA that was synthesised using Akirin-specific PCR products, that were amplified from cDNA which was reverse-transcribed from RNA isolated from 1-day-old female adult *An. arabiensis* mosquitoes, and electrophoresed on a 1% agarose gel in TAE (110V, 75-minutes). M: O'GeneRuler™ 100bp DNA ladder, ready to use (ThermoFisher Scientific, SM1143), which was used, as opposed to the RNA ladder, due to the double-strand nature of the *in vitro* transcribed product. Lane 1-3: *in vitro* transcribed Akirin-specific dsRNA. Lane 4: no template control.

### 4.3.2 Determination of the relative gene expression of *akirin* in *An. arabiensis* post-RNAi inoculation

*Anopheles arabiensis* treated with Akirin-specific dsRNA showed a significant reduction in *akirin* expression, by a mean factor of 0.76 (S.E. range is 0.65-0.88) ($p$=0.02), when compared to the negative control samples (Figure 4.5). As expected, no significant change was detected amongst the control treatments, confirming that the change in relative expression was gene-specific. The change in *akirin* expression was also assessed in the samples treated with Akirin-specific siRNA. Although the Akirin-specific siRNA treated samples showed a reduction in *akirin* expression, by a mean factor of 0.82, (S.E. range is 0.65-0.99) ($p$=0.06) when compared to the negative control samples, the change in expression was not statistically significant at a 95% confidence interval (Letinić *et al*., 2020).

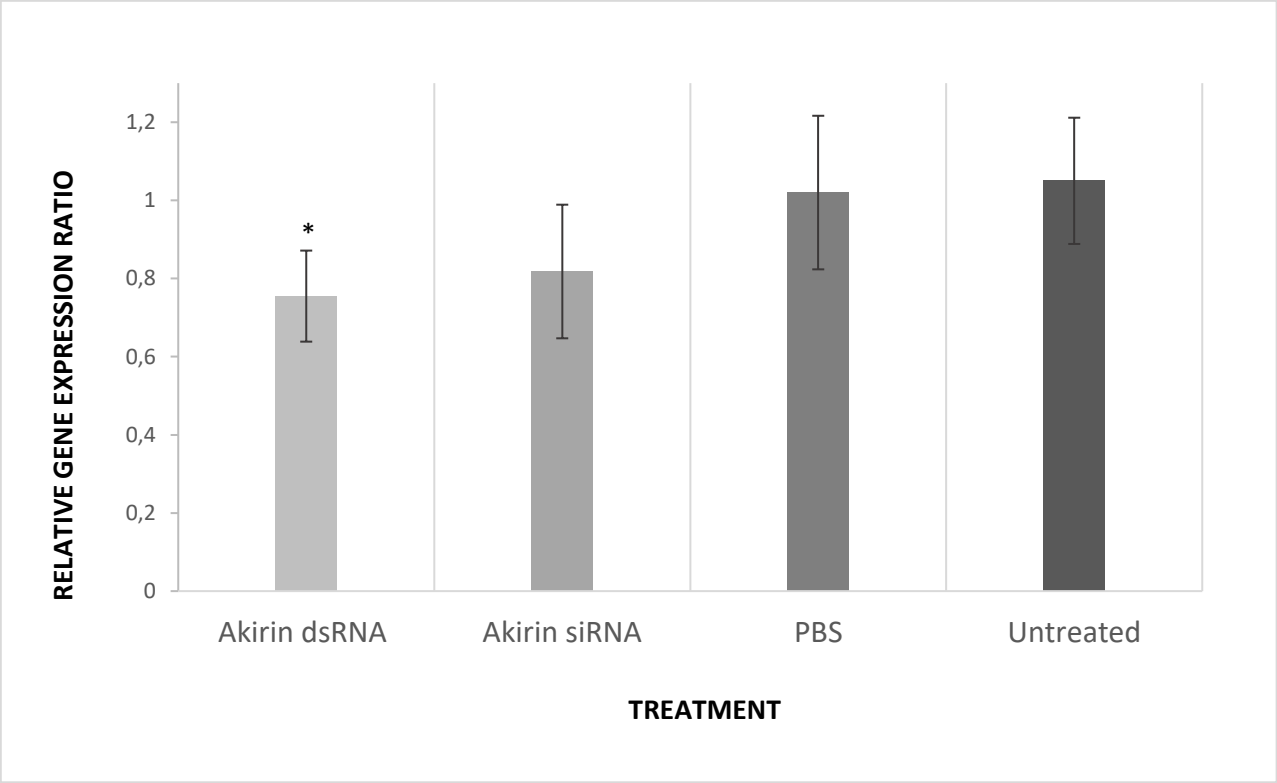


**Figure 4.5** The change in relative a*kirin* expression assessed three-days post-RNAi treatment. The relative expression of *akirin* was significantly downregulated by a mean factor of 0.76 ($p$=0.02), when comparing the relative expression ratios of *akirin* between the Akirin-specific dsRNA treated samples and the Mouse-β2m-specific siRNA treated samples (negative control samples). The relative expression of *akirin* remained unchanged, when comparing the expression ratios of the negative control samples to the PBS treated samples ($p$=0.78) and cold immobilised, untreated samples ($p$=0.83). The asterisk shows a significant change from the negative control treatment, while the error bars on the graph are indicative of the S.E. between the treatment replicates.

### 4.3.3 Evaluation of the effect of *akirin* suppression, by RNAi, on *An. arabiensis* lifetable parameters

Vector fecundity and fertility were assessed, to determine whether *akirin* knockdown had an impact on *An. arabiensis* reproduction (Figure 4.6). *Anopheles arabiensis* treated with Akirin-specific siRNA and Akirin-specific dsRNA had a 1.3-fold (17%) and 1.4-fold (25%) decrease in fecundity respectively, when compared to the control treatments (one-way ANOVA: $p<0.01$, F=16.5, DF=4). The control treatments had a mean fecundity of approximately 110 eggs laid per female mosquito, while those treated with Akirin-specific siRNA and Akirin-specific dsRNA had mean fecundity of 91 and 82 eggs laid per female, respectively. *Anopheles arabiensis* treated with Akirin-specific siRNA and Akirin-specific dsRNA had a further 1.2-fold (19%) and 1.4-fold (26%) decrease in fertility respectively, when compared to the mean of the control treatments (one-way ANOVA: $p<0.01$, F=63.4, DF=4). The control treatments had a mean hatch rate of 92%, while those treated with Akirin-specific siRNA and Akirin-specific dsRNA had a mean hatch rate of 72% and 65% respectively (Letinić *et al*., 2020).

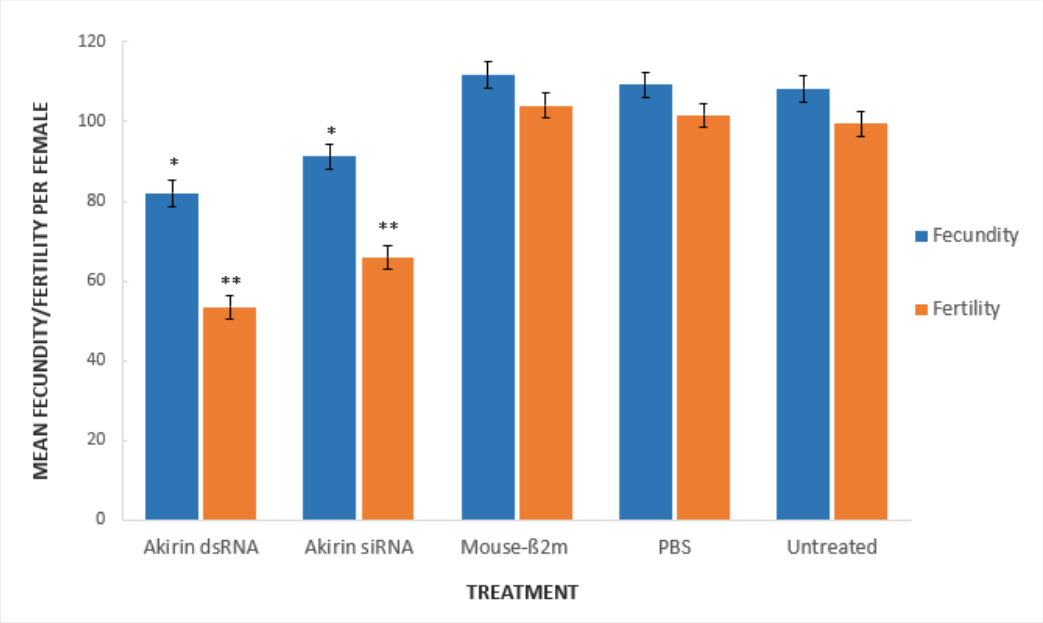


**Figure 4.6** The mean vector fecundity (blue), and fertility (orange) per female, per treatment. The *akirin* knockdown mosquitoes had a reduced mean fecundity by 17-25% (one-way ANOVA: $p<0.01$, F=16.5, DF=4), and reduced mean fertility by 23-29% (one-way ANOVA: $p<0.01$, F=63.4, DF=4), when compared to the control treatments. This was a 19-26% decrease in hatch rate percentage. No significant change in fecundity/fertility was observed between the control treatments. The asterisks indicate a significant change from the control treatments, while the error bars on the graph are indicative of the S.E. between the treatment replicates.

Vector longevity was significantly reduced in the *akirin* knockdown female *An. arabiensis* mosquitoes when compared to the control (Figure 4.7). The control treatments had a mean survival time of 28-days, while the mosquitoes treated with Akirin-specific siRNA and Akirin-specific dsRNA had a mean survival time of 20-days and 15-days post-inoculation, respectively. This was a 29% and 46% decrease in mean survival time when compared to the control treatments. At 15-days post-inoculation, *An. arabiensis* treated with Akirin-specific siRNA had a mortality of 35%, while the mosquitoes treated with Akirin-specific dsRNA had a mortality of 52%. This was significantly higher than the controls (Log-rank: $\chi^2$=78, $p$<0.01, DF=4), as the mosquitoes treated with Mouse-β2m-specific siRNA and PBS had a mortality of 20%, and the untreated mosquitoes had a mortality of 12%. *Anopheles arabiensis* treated with Akirin-specific siRNA and Akirin-specific dsRNA reached 100% mortality 33-days and 25-days post-inoculation respectively, while the control treatments reached 100% mortality after 47-days. When compared to the control treatments, the mosquitoes treated with Akirin-specific siRNA had a 32% decrease in longevity overall, while the mosquitoes treated with Akirin-specific dsRNA had a 48% decrease in longevity in total (Log-rank: $\chi^2$=154, $p$<0.01, DF=4) (Letinić *et al*., 2020).

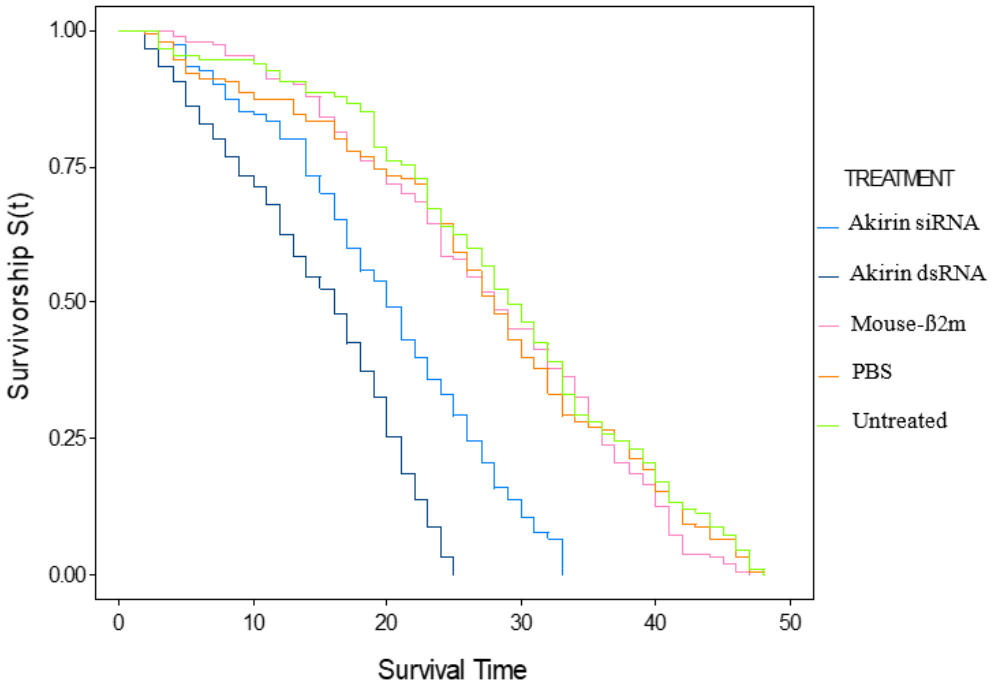


**Figure 4.7** Kaplan-Meier survival analysis of *akirin* knockdown, Mouse-β2m negative control (pink), PBS handling control (orange), and untreated control (green) mosquitoes, post-RNAi treatment. Female *An. arabiensis* treated with Akirin-specific siRNA (light-blue) and Akirin-specific dsRNA (dark-blue) reached 100% mortality 15-23 days before the control treatments, which conveyed a 32-48% decrease in vector longevity (Log-rank: $\chi^2$=154, $p$<0.01, DF=4).

## 4.4 Discussion

Following successful transcription, mRNA is translated into an amino acid chain. Cells use a variety of mechanisms to cause or prevent the degradation of these mRNA molecules. This naturally occurring mechanism allowed RNAi gene manipulation to be performed in *An. arabiensis* by targeting endogenous mRNA, using dsRNA. Two different knockdown molecules (dsRNA and siRNA) were used to target Akirin mRNA in *An. arabiensis.* However, regardless of the molecule used to repress *akirin* expression, a statistically significant change in vector fecundity, fertility and longevity were observed. This was consistent with the results obtained when *akirin* was downregulated in *An. coluzzii,* where vector fecundity was reduced by 52%, and vector survival was reduced by 14% (da Costa *et al*., 2014).

The *in vitro* transcribed exogenous dsRNA was more efficient in repressing *akirin* expression in *An. arabiensis* than the commercially available exogenous siRNA. This is likely due to improved absorption uptake of the dsRNA (Saleh *et al*., 2006), as well as asymmetry in the assembly of the RNAi enzyme complex (Schwarz *et al*., 2003). Although less efficient, deleterious effects were still apparent when assessing lifetable parameters in the mosquitoes treated with Akirin-specific siRNA. This suggested that *akirin* expression may have recovered after being repressed, however, its downregulation still had downstream physiological effects in *An. arabiensis* (Wang *et al.,* 2013).

Mosquitoes treated with Akirin-specific dsRNA had a 46% reduction in mean survival time in relation to the controls. The control treatments had a mean survival time of 28-days, while the mosquitoes treated with Akirin-specific dsRNA had a mean survival time of 15-days post-inoculation (Letinić *et al*., 2020). This was a significant finding since *Anopheles* mosquitoes can transmit malaria parasites 14-days post-gametocyte infection (Bray and Garnham, 1982). Suppressing half of the vector population prior to the completion of the extrinsic incubation period would have a substantial impact on malaria transmission, as the female mosquito needs to survive longer than 14-days post-gametocyte infection, to transmit the parasite.

The downregulation of *akirin* impairs the gene expression of several antimicrobial peptide-coding genes (Bonnay *et al*., 2014). As aforementioned, it is possible that this disrupts the bacterial homeostasis in the mosquitoes midgut, and weakens the innate immune defence required for survival upon immune challenge (Ferrandon *et al.,* 2007; Goto *et al.,* 2008; Nowak

*et al.,* 2012; Mukherji *et al.,* 2013; Bonnay *et al*., 2014; Debalke *et al*., 2019). In addition to causing a reduction in mosquito longevity, a disruption in midgut homeostasis may reduce the availability of nutrients after taking a blood meal. This would cause the developing follicles to be reabsorbed, resulting in reduced vector ovipositioning (Suneja *et al.,* 2003). However, Akirin is also known to play a critical role in processes unrelated to immunity, which may be involved in reproduction (Goto *et al.,* 2008; Canales *et al.,* 2009). Regardless of the underlying mechanism, *akirin* knockdown in *An. arabiensis* resulted in a 17-25% decrease in vector fecundity, and a 23-29% decrease in vector fertility. This attested the potential of targeting Akirin for *An. arabiensis* vector control, using RNAi.

Although the use of nano-injection is efficient for the study of functional genomics, this technique is not pertinent for the management of insects in the field. To achieve the same deleterious phenotypes in wild populations of *An. arabiensis* as those observed in the lab, dsRNA must be delivered to *An. arabiensis* free of degradation, using field feasible applications. Sugar-feeding constitutes a vital source of sustenance for adult mosquitoes of both sexes (Foster, 1995). Within hours of emergence, both male and female mosquitoes take a sugar-meal (Foster, 1995). This behaviour could be used to administer Akirin-specific dsRNA to *An. arabiensis* via the oral exposure route.

In nature, the most common sources of sugar for mosquitoes are floral-nectar, extrafloral-nectar, honeydew, tree sap, damaged fruit, and portions of damaged leaves (McCrae *et al.,* 1969; Foster, 1995). These plants could potentially be engineered to express hairpin RNAs that are functionally equivalent to that of the linear Akirin-specific dsRNA. The hairpin RNAs would consequently be ingested by *An. arabiensis* when taking a sugar-meal, which would result in the downregulation of *akirin* expression in the malaria vector. Transgenic plants have previously been used to downregulate gene expression in several insect species, through the delivery of dsRNA (Baum *et al*., 2007; Zha *et al*., 2011). However, this application has not yet been evaluated in Culicidae, and thus would need further investigation.

Alternatively, dsRNA could potentially be delivered to *An. arabiensis* in the form of attractive-toxic sugar baits (Qualls *et al.,* 2014; Zhu *et al.,* 2015). Akirin-specific dsRNA could be mixed with a sucrose-meal or an artificial blood-meal, and administered to *An. arabiensis* in bait stations near breeding sites. This would target the vector population by reducing the mean vector longevity, which has the capacity to aid in reducing malaria transmission. Large

quantities of the Akirin-specific dsRNA could be produced using an *E. coli expression* system, which lacks the dsRNA-specific endonuclease, RNase III (Timmons *et al.*, 2001). However, the stability of Akirin-specific dsRNA, in combination with sucrose-meals or artificial blood-meals, would need to be assessed. Additionally, to use this application the downregulation of *akirin* expression would need to be confirmed via the oral exposure route.

Topical applications of dsRNA have also previously been used to effectively mediate RNAi in Culicidae, despite the physical barrier of the exoskeleton (Pridgeon *et al.*, 2014). This was demonstrated when topical application bioassays were performed on the dorsal thorax of adult female *Aedes aegypti,* using acetone as a carrier for the delivery of dsRNA. Subsequently, the expression of *IAP1* was successfully downregulated (Pridgeon *et al.*, 2014). This strategy could therefore be used to essentially develop a molecular insecticide that could target Akirin in *An. arabiensis.*

Although future research is needed to determine whether Akirin can be targeted in wild populations of *An. arabiensis* using RNAi, the effect of *akirin* knockdown on vector fecundity, fertility, and longevity, has made Akirin in *An. arabiensis* a valuable candidate to use as a recombinant vaccine. Furthermore, the opportunistic feeding behaviour characteristic of *An. arabiensis* females, as well as a significant reduction in *An. arabiensis* lifetable parameters observed when targeting Akirin, provides the impetus to evaluate the use of recombinant Akirin vaccines in this major African malaria vector.

This study, therefore, shows that RNAi can be used to repress *akirin* expression in *An. arabiensis.* However, RNAi applications would need to be further developed to use this tool for *An. arabiensis* vector control in the field. It also shows that reducing *akirin* expression in *An. arabiensis* can cause a significant decrease in vector longevity, fecundity, and fertility. A reduction in vector mean survival, as well as reproduction, would have a significant impact on residual malaria transmission, in countries like South Africa, due to a reduction in vector abundance.

# CHAPTER 5: IMMUNISATION

**Objective:** Assess the effect of recombinant Akirin vaccines on *An. arabiensis*, to determine its potential as a vector control tool.

The work described in Chapter 5 has been submitted for publication (Appendix F): **Letinić, B.D., Contreras, M., Dahan-Moss, Y., Linnekugel, I., de la Fuente, J. and Koekemoer, L.L.**, 2020. Characterising the effect of three different recombinant Akirin vaccines on *Anopheles arabiensis. Parasites & Vectors*, (submitted and under review).

## 5.1 Introduction

The concept of vaccinating agricultural animals with recombinant vaccines for the control of endo- and ectoparasitic infestations was first demonstrated in *Dermacentor andersoni.* Infestations of the blood-feeding ectoparasite were reduced after feeding on cattle that had been vaccinated with crude extracts of gut antigens from these arthropods (Allen and Humphreys, 1979). These gut antigens caused antigen-specific antibodies to develop within the immunised host. The arthropod vectors ingested these antibodies when feeding on immunised hosts, which interacted with and disrupted the function of the target antigen in the arthropod vector (Willadsen *et al.*, 1989). This resulted in physiological changes that affected the vector's biology (Elvin and Kemp, 1994).

The first commercially available arthropod vaccines, namely Gavac™ and TickGARD$^{PLUS}$ were subsequently manufactured and distributed after the *Rhipicephalus microplus* gut antigen (BM86) was used to control tick infestations, through cattle immunisation (Rand *et al.*, 1989; Willadsen *et al.*, 1989; Rodríguez *et al.*, 1994; de la Fuente *et al.*, 1998). It was since proposed that immunisation, using evolutionary conserved protective antigens, could be used to develop multi-target vaccines directed at the control of several vector species (de la Fuente and Kocan, 2003; Canales *et al.*, 2009). However, the limiting step in the development of this vaccine is the discovery of such antigens (de la Fuente and Kocan, 2003).

The evolutionary conserved protective antigen, Subolesin, was found in *I. scapularis* using expression library immunisation (Almazán *et al.*, 2003; Almazán *et al.*, 2005; de la Fuente *et al.*, 2006). The ingestion of recombinant Subolesin antibodies caused several deleterious

effects in engorged ticks, and included diminished vectorial capacity, reduced oviposition, and reduced survivorship (Almazán *et al.,* 2005; Almazán *et al.,* 2010). Feeding capabilities were also significantly reduced, resulting in a decline of tick mass (de la Fuente *et al.,* 2006). Additionally, developmental abnormalities were observed, such as the failure of larvae to undergo metamorphosis into nymphs, as well as tissue damage in various tick species (Almazán *et al.,* 2005; de la Fuente *et al.,* 2010). Since Akirin is the insect ortholog of Subolesin, recombinant Akirin vaccines could be used to target mosquitoes.

Preliminary immunisation studies were evaluated on *An. atroparvus, Ae. caspius*, and *Culex pipiens* mosquitoes (Canales *et al.,* 2009). Immune serum was acquired from New Zealand white rabbits that were vaccinated with recombinant Akirin from *Ae. albopictus.* The hyperimmune serum was administered to the various mosquito species via a solution embedded in cotton wool, to ascertain the effect of antigen-specific antibodies on mosquito lifetable parameters. The ingestion of the antigen-specific antibodies decreased mosquito survivorship (Canales *et al.,* 2009), which provided the impetus needed for subsequent recombinant *Ae. albopictus* Akirin immunisation studies.

Mice were subsequently vaccinated with recombinant *Ae. albopictus* Akirin (Akirin$^{albopictus}$), to evaluate the effect of antigen-specific antibodies on *Ae. albopictus, Ae. aegypti* and *An. coluzzii* lifetable parameters: however, the results from these investigations varied (Table 5.1) (Moreno-Cid *et al.,* 2010; da Costa *et al.,* 2014). The ingestion of Akirin$^{albopictus}$ antibodies diminished *Ae. albopictus* and *An. coluzzii* reproductive capacities, yet it failed to reduced vector survivorship (Moreno-Cid *et al.,* 2010; da Costa *et al.,* 2014). On the contrary, the ingestion of Akirin$^{albopictus}$ antibodies failed to reduce both vector survivorship and reproductive capacities in *Ae. aegypti* (Moreno-Cid *et al.,* 2010). The difference in vaccine efficacy was attributed to the physiological differences between the various species (Moreno-Cid *et al.,* 2010).

Another antigen was subsequently evaluated for the control of *Ae. albopictus* mosquito populations. The Q38 antigen was a chimera that was designed by combining protective epitopes from *I. scapularis* Subolesin and *Ae. albopictus* Akirin (Moreno-Cid *et al.,* 2013). Ingestion of Q38 antigen-specific antibodies reduced *Ae. albopictus* reproductive capacities as well as survivorship, demonstrating the possibility of using Q38 as a multi-target vaccine (Moreno-Cid *et al.,* 2013).

**Table 5.1** Akirin immunisation studies previously conducted on various mosquito species.

| Reference | Species of Mosquito | Recombinant Protein | Feeding Source | Immunisation Schedule | Antigen Dosage (µg/dose) | Mosquito Sample Size | Feeding (min) | Phenotypic Evaluation | Results |
|---|---|---|---|---|---|---|---|---|---|
| Moreno-Cid *et al.*, 2010 | *Ae. albopictus* | Akirin *albopictus* | Five female 5-week-old balb/c mice | Immunized three times at weeks 0, 3 and 6 with 1mL doses | 50 | 20 | 30 | **Oviposition:** Eggs counted 7-days post-feeding<br><br>**Mortality rate:** Evaluated 7-days post-feeding | Oviposition was reduced by 17% when compared to the controls however, survival was not affected. |
| Moreno-Cid *et al.*, 2010 | *Ae. aegypti* | Akirin *albopictus* | Four female 8–10 weeks old balb/c mice | Immunized three times at weeks 0, 4 and 6 with 1mL doses | 50 | 20 | 15 | **Oviposition:** Eggs counted 7-days post-feeding<br><br>**Mortality rate:** Evaluated 7-days post-feeding | Vector survival and oviposition not effected after having fed on vaccinated mice. |
| Moreno-Cid *et al.*, 2013 | *Ae. albopictus* | Q38 | Six female 5-week-old balb/c mice | Immunized three times at weeks 0, 3 and 6 with 0.2mL doses | 25 | 40 | 30 | **Oviposition:** Eggs counted 15-days post-feeding Eggs incubated 72-hours in water to evaluate hatchlings<br><br>**Mortality rate:** Evaluated 10-days post-feeding | Reduced survival by 12%, and oviposition by 28%, when compared to controls. |
| da Costa *et al.*, 2014 | *An. coluzzii* | Akirin *albopictus* | Female 5-week-old balb/c mice | Immunized four times with 0.1mL doses given 2 weeks apart | 20 | 30 | 30-45 | **Oviposition:** Eggs counted 7-days post-feeding<br><br>**Mortality rate:** Evaluated 8-days post-feeding | Vector fecundity was significantly reduced by 37% when compared to controls Survival was not affected. |

The opportunistic feeding behaviour characteristic of *An. arabiensis* females, as well as a significant reduction in *An. arabiensis* lifetable parameters observed when targeting Akirin, using RNAi, provided the impetus to evaluate the use of recombinant Akirin vaccines in this major African malaria vector. To establish whether Akirin could represent a novel target for the control of *An. arabiensis*, the efficacy of recombinant Akirin$^{albopictus}$ (Moreno-Cid *et al.*, 2010), and recombinant Q38, (Moreno-Cid *et al.*, 2013), were evaluated on *An. arabiensis* lifetable parameters. However, *Aedes* are vectors for diseases unrelated to malaria, namely dengue fever, yellow fever, chikungunya, and Zika virus among many others. Therefore, an additional genera-specific recombinant antigen was evaluated on *An. arabiensis*, namely recombinant Akirin, from *An. arabiensis* (Akirin$^{arabiensis}$).

*Anopheles arabiensis* will readily feed on animal as well as human hosts therefore, it was possible to target Akirin by exploiting the natural feeding behaviour of this vector using recombinant Akirin vaccines. The recombinant Akirin vaccines were expressed and administered to balb/c mice, to elicit an antigen-antibody response in these reservoir hosts. Mice injected with a vaccine containing PBS and adjuvant were used as a negative control. The antibody titres in the mice were assessed post-immunisation, after which *An. arabiensis* were permitted to take a blood meal. The antibody titres were assessed in *An. arabiensis* which had taken a blood meal, to determine whether the antigen-specific antibodies were ingested by the mosquitoes.

Once ingested, the antibodies would cross the cell membrane and interacted with Akirin in the cytoplasm (de la Fuente *et al.*, 2011; de la Fuente *et al.*, 2013). This would prevent Akirin from translocation to the nucleus, which would disrupt the biological functions of Akirin within *An. arabiensis*. Vector fertility, fecundity, and survival were subsequently assessed in *An. arabiensis*, to determine whether targeting Akirin in *An. arabiensis*, while feeding on animals, could provide an additional intervention to the current vector control activities.

## 5.2 Methods

### 5.2.1 Formulation of recombinant Akirin vaccines

Primers were designed to amplify the coding region of Akirin (GenBank: KU973613) (Figure 5.1). The Akirin forward primer (5′CACCATGGCGTGCGCAACGTTA3′) was 22bp in length, with a melting temperature of 68°C and a GC content of 59%. The forward primer was designed to facilitate directional cloning in the pET TOPO® vector (Invitrogen, K101-01), by including the "CACC" overhang sequence (red), which preceded the initial start codon (ATG) (blue). These four nucleotides paired with the overhang sequence, "GTGG", in the pET101/D TOPO® vector. The Akirin reverse primer (5′GGAGAGGTAACTTGGCGCTGCTTC3′) was 24bp in length, with a melting temperature of 67°C, and a GC content of 58%. The reverse PCR primer was designed to exclude the native stop codon, which allowed the PCR product to be cloned in frame with the V5 epitope, and the 6xHis-tag, in the TOPO® vector (Invitrogen, K101-01).

5'CACCATGGCGTGCGCAACGTTAAAACGTTCGCTGGACTGGGAAAGCCTCAACC
AGCGGCCGACCAAGCGGCGAAGATGTCACCCATTCGGCAGCCCCAGCCAGACCG
CGTCTGCCTCCTCCTCCTCCGCTTCCCCGTCCGGCTCATCCTCCACATCCGTTGCA
GCGGCCGCTGCCGCCGCGTCGATGCGCGTCATGGAACCGAAACCGTCGCCTTTTG
CAGAAGCCACCTGTTCCAAGCTAACACCAGAAAAAATGGCACAAAATATCACGG
AGGAAATCAAAAGACTGCACCGCAGGAAGCAACTCACCTTCAACAGCCACAACT
TCGAACGCATGCAGGACTCGGAATCGAGCGGCTCCGAAATGGGCCCGGACAGTC
CCCGCCGACCAGACAGCCCTCCTAGCATGGTGAAAAATCCGGAAAAAGCCCTGT
TCACCTTCAAGCAGGTGCAAATGATCTGTGAACGTATGCTGAAAGAACGAGAGG
ATGCCTTGCGGGAGCAGTATGATGCCGTGCTGACCACTAAACTAGCCGAACAAT
ATGATGCATTTGTGAAGTTCACATACGACCAAATACAGAGACGTTACGAAGCAG
CGCCAAGTTACCTCTCCTAA3'

**Figure 5.1** Gene construct showing the location of each primer (underlined) on the coding sequence of Akirin in *An. arabiensis* (GenBank: KU973613), which were used to amplify a 613bp amplicon. The forward primer included the transcription start codon (ATG) (blue) of Akirin, which was preceded by the "CACC" overhang sequence (red), that facilitated directional cloning in the pET TOPO® vector. The reverse primer was designed to exclude the native stop codon (grey), which allowed the PCR product to be cloned in frame with the V5 epitope and the 6xHis-tag, in the TOPO® vector, to express recombinant Akirin*arabiensis* protein.

RNA was extracted from *An. arabiensis* mosquitoes using Tri Reagent (Sigma-Aldrich, T9424). The isolated RNA was purified using the RNeasy® MinElute™ clean-up kit (Qiagen, 74204). The NanoDrop™ spectrophotometer was used to assess the concentration of the eluted purified RNA, and the integrity of the RNA was assessed using a 1% non-denaturing agarose gel in TBE, which was electrophoresed for 30-minutes at 70V. The RNA was reverse-transcribed into cDNA, and amplified using the Access RT-PCR system (Promega, A1250).

The RT-PCR reaction was assembled using 1µM Akirin forward primer, 1µM Akirin reverse primer, 1mM MgSO4, 0.2mM dNTP mix, 0.1U *Avian Myeloblastosis Virus* (*AMV*) reverse-transcriptase, and 0.1U *Thermus flavus* (*Tfl*) DNA polymerase. The sample was made up to a final volume of 50µL using nuclease-free water, and incubated at 45°C for 45-minutes. This allowed for first-strand cDNA synthesis to take place. The cDNA sample was heated to 94°C, for 2-minutes, to inactivate the *AMV* reverse-transcriptase enzyme. Once inactivated, 40 PCR cycles were conducted to amplify Akirin (94°C for 30-seconds, 58°C for 1-minute, 68°C for 2-minutes), with a final PCR extension at 68°C for 10-minutes.

The Akirin PCR product was purified using the MinElute® PCR purification kit (Qiagen, 28004). Binding buffer was added to the RT-PCR product (5:1), and transferred to the supplied MinElute® column. The MinElute® column was placed into a 2mL collection tube and centrifuged to bind the DNA to the column (17,900 x *g*,1-minute). The MinElute® column was washed with 750µL wash buffer (17,900 x *g*,1-minute), and placed into a new 1.5mL microcentrifuge tube. The purified RT-PCR product was eluted in 10µL of elution buffer, and quantified using the NanoDrop™ spectrophotometer. The integrity of the purified RT-PCR product was subsequently assessed using a 1% agarose gel in TAE, which was electrophoresed for 75-minutes at 100V, and was sent for sequencing. Sequencing was conducted by double-stranded dye-termination cycle sequencing (Secugen SL, Madrid, Spain).

The purified RT-PCR product was cloned into the pET101/D -TOPO® vector (Invitrogen, K101-01). The TOPO® cloning reaction was assembled using a 0.5:1 molar ratio of RT-PCR product to TOPO® vector. A salt solution, comprised of 24mM NaCl and1.2mM $MgCl_2$, was added to the reaction. The reaction was made up to a final volume of 5µL, using nuclease-free water. The TOPO® cloning reaction was incubated for 5-minutes, at room temperature, and returned to the ice to conduct transformation. The pET TOPO® construct was transformed into One Shot® TOP10 chemically competent *E. coli* cells (Invitrogen, C6010), using 3µL of

TOPO® cloning reaction. After a 30-minute incubation on ice, the cells were heat pulsed for 30-seconds at 42°C. The cells were immediately returned to the ice, where room-temperature SOC media (super optimal broth with catabolite repression) was added to the cells (250µL), to allow for recovery post-transformation (200rpm, 37°C, 1-hour).

The transformed cells were spread (200µL) on Lysogeny broth (LB) agar plates, containing 50µg/mL ampicillin. After overnight incubation (37°C), a total of five colonies were selected from the plates. The selected colonies were assessed for the Akirin insert using PCR. PCR was conducted using 1µM Akirin forward primer, 1µM Akirin reverse primer, and 12.5µL PCR SuperMix (Invitrogen, 10790-020), which was made up to a final volume of 25µL using nuclease-free water. The samples were heated to 94°C for 2-minutes, followed by 40 PCR cycles (94°C for 30-seconds, 58°C for 1-minute, 68°C for 2-minutes), with a final PCR extension at 68°C for 10-minutes. The PCR products were quantified using the NanoDrop™ spectrophotometer, and assessed using a 1% agarose gel in TAE, which was electrophoresed for 75-minutes at 100V, and sent for sequencing.

The GenJet™ plasmid miniprep kit (Invitrogen, K0502) was used to isolate and purify the plasmid DNA from the selected colonies. The selected colonies were inoculated in 2mL of LB media containing 50µg/mL of ampicillin. The inoculated media was propagated by shaking at 250rpm (16-hours, 37°C). The bacterial cells were harvested by centrifugation (6,800 x *g*, 2-minutes), after which the supernatant was discarded. The harvested cells were resuspended in resuspension solution (250µL) and mixed using a vortex. Lysis solution (250µL) was added to the suspension, and mixed by inversion. Neutralization solution (350µL) was added to stop the lysis process. The samples were centrifuged to collect the cell debris (5-minutes, 13,000 x *g*). The supernatant was transferred to the supplied GeneJet™ spin column and centrifuged for 1-minute at 11,000 x *g*. The column was washed twice with wash solution (500µL), and placed in a new microcentrifuge tube. The purified plasmid was eluted in elution buffer (10µL).

The purified plasmid was transformed into recombinant *E. coli* BL21 Star™(DE3) One Shot® cells (Invitrogen, C6010-03). Plasmid DNA (10ng) was added to one vile of cells and incubated for 30-minutes on ice. After incubation, the cells were heat-pulsed for 30-seconds, at 42°C, without shaking, and immediately transferred back onto the ice. Room-temperature LB media was added to the cells (250µL), and incubated at 37°C for 30-minutes with shaking (200rpm), to allow for cell recovery to take place. The transformation reaction was inoculated into 10mL

of LB media, containing 50μg/mL ampicillin and 0.5% glucose (CONDA, 21528), and incubated overnight (37°C, 200rpm) to propagate cells.

After overnight incubation, 1mL of culture was removed from the propagated LB media, to create a bacterial glycerol stock. The *E. coli* cells, containing the plasmid with recombinant Akirin$^{arabiensis}$, were added to a 50% glycerol solution (1:1) and stored at -80°C for future use. Working glycerol stocks of *E. coli* cells, containing recombinant Akirin$^{albopictus}$ and Q38 plasmids, were used to express these recombinant proteins. These recombinant proteins were initially expressed by Canales *et al.* (2009) and Moreno-Cid *et al.* (2013), and are maintained as glycerol stocks at the Instituto de Investigación en Recursos Cinegéticos, IREC, Ciudad Real, Spain. *Anopheles arabiensis* Akirin (612bp) and *Ae. albopictus* Akirin (603bp) have a sequence similarity of 78%, while *An. arabiensis* Akirin and Q38 (657bp) have a sequence similarity of 49.1%. Using the *E. coli* expression system (Contreras *et al.*, 2017), glycerol stocks were inoculated into 10mL of LB media, containing 50μg/mL ampicillin and 0.5% glucose (CONDA, 21528), and were incubated overnight (37°C, 200rpm) to propagate cells (Figure 5.2). After overnight incubation, 9mL of culture was transferred into a flask containing 300mL of LB media (50μg/mL ampicillin, 0.5% glucose). The culture was propagated for a further 2-hours (37°C, 200rpm) to reach an $OD_{600nm}$ = 0.4.

Isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, 367-93-1) was subsequently added to the propagated culture, to a final concentration of 0.5mM. The culture was incubated for an additional 4 to 6-hours (37°C, 200rpm), to induce heterologous protein expression. Once propagated, the optical density of the culture was measured using a spectrophotometer ($OD_{600nm}$ = 1). Centrifugation was conducted to harvest the bacterial cells (3,900 x *g*, 15-minutes, 4°C).

Harvested cells (3g) were resuspended in 15mL of lysis buffer (50mM potassium phosphate, pH 7.8, 400mM NaCl, 100mM KCl, 7M Urea, 10mM Imidazole), which contained a protease inhibitor (Roche, 04693132001). The resuspended cells were mechanically disrupted by sonication (30% amplitude, 30-second on/off pulses) using the Ultrasonic Homogenizer (Bandelin Sonopuls, Model MS73). After sonification, centrifugation was conducted at 15,000 x *g* (15-minutes, 4°C). The supernatant, containing the soluble recombinant protein, was transferred to a new tube, and the pellet was discarded.

The recombinant proteins were purified by nickel affinity chromatography, using a 1mL HisTrap™ FF column, which was mounted on the ÄKTA-FPLC system (GE Healthcare, ÄKTA prime plus). Fractions were eluted in elution buffer (50mM $KH_2PO_4$ pH7.8, 400mM NaCl, 100mM KCl, 7M Urea, 500mM Imidazole). Eluted fractions containing the purified protein were collected for dialysis.


**Figure 5.2** An overview of the recombinant protein expression process using an *E. coli* expression system. 1: *Escherichia coli* cells that contained the recombinant proteins were propagated in LB media, overnight. 2: Propagation was upscaled by adding the *E. coli* cells into a flask containing additional LB media, after which IPTG was added to induced heterologous expression. 3: The bacterial cells were harvested by centrifugation. 4: The harvested cells were mechanically disrupted by sonification. 5: The supernatant containing the recombinant protein was collected by centrifugation. 6: The recombinant proteins were purified by nickel affinity chromatography.

Dialysis was conducted to reduce the salt concentration of the eluted fractions, which contained the purified recombinant proteins. The eluted fractions were transferred into a cellulose dialysis tubing membrane (Sigma-Aldrich, 3110), and incubated in 1x PBS (137mM NaCl, 2.7mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$ pH 7.4) for 12-hours, at 4ºC. The purified recombinant proteins were quantified using the Pierce™ BCA protein assay kit (ThermoFisher Scientific, 23225). Total protein quantification was determined using bicinchoninic acid

colourimetric detection. The proteins of unknown concentrations were compared to a standard curve of known concentration. The standard curve was prepared using a two-fold serial dilution (20mg/mL) of bovine serum albumin (BSA). Samples were prepared in a hard-well plate, using 200µL of working reagent to 10µL of recombinant protein (2:1). The working reagent was prepared by adding 50 parts of reagent A to 1 part of reagent B. After a 30-minute incubation (in the dark, 37°C), total protein quantification was determined using a plate reader (562nm).

Recombinant protein expression was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The Spectra™ multicolour broad range protein ladder (ThermoFisher Scientific, 26634) was used to determine the molecular weight of the recombinant proteins. Total protein (10µg) was loaded on a 12% SDS-PAGE precast gel (C.B.S. Scientific, BK01212-10), and electrophoresed for 1-hour at 180mA. The gel was either stained with Blue BANDit™ stain (VWR Life Science, K217) for 1-hour or used for Western blot analysis. After stained, the SDS-PAGE gel was washed overnight in distilled water, and visualized using the gel doc system.

For Western blot analysis, the SDS-PAGE gel was was transferred to a nitrocellulose membrane, and the membrane was blocked for 2-hour, at room temperature, with 5% BSA. The membrane was washed four times with Tris-buffered saline (TBS; 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Tween 20) and incubated with anti-$His_6$ from mouse (Sigma-Aldrich, 11922416001) at a 1:500 dilution in TBS. The membrane was incubated overnight at 4°C and washed four times with TBS. The membrane was then incubated with an anti-mouse IgG-horseradish peroxidase (HRP) conjugate (Sigma-Aldrich, AP308P) diluted to 1:1000 in TBS with 3% BSA. The membrane was washed five times with TBS and finally developed with TMB (3,3´, 5,5´- tetramethylbenzidine) stabilised substrate for HRP (Promega, W4121) according to the manufacturer recommendations.

The recombinant proteins were concentrated to a final concentration of 250µg/mL in PBS. The recombinant proteins were emulsified with 1.5mL of adjuvant (Montanide™, ISA 50 V2), to formulate recombinant vaccines, which were administered to balb/c mice. A vaccine consisting of PBS and adjuvant was used as a negative control. The researchers were blinded from the vaccine treatment until the end of the study.

### 5.2.2 Performance of recombinant Akirin immunisation on balb/c mice and assessment of the antibody titres

Five-week-old balb/c mice were vaccinated intraperitoneally, using a double-blind approach, where each treatment was given a code and the identity of the code was not revealed to the researchers until the end of the experiment. Therefore, mice were either vaccinated with recombinant protein (25µg/dose), or the control consisting of saline and adjuvant. Each mouse received three doses of vaccine, which were administered 2-weeks apart.

The balb/c colony was maintained at the South African Vaccine Producers (SAVP) animal unit of the NICD, at a temperature range of 21˚C (±2˚C), with a 12-hour day/night cycle. The mice (1 per treatment, 4 treatments, 3 replicates, n=12) were housed in standard laboratory cages, on a bed of pine shavings, and added shredded paper for enrichment: bedding was changed twice a week. The mice were sustained on a diet consisting of commercial mouse cubes (Epol®, 4700) as well as water *ad-lib*. The mice did not suffer any ill effects during this study. Mice were euthanised upon experimental completion. Animal handling, injections, and veterinary procedures were strictly conducted by trained South African Veterinary Council (SAVC) registered staff. Animal ethics was reviewed and approved by the University of the Witwatersrand (WITS) Animal Ethics Committee (AEC) (AESC: 2017-010-72B), as well as the National Health Laboratory Service (NHLS) AEC (AESC: 001-19). See Appendix C.

The immunised mice were used to provide female *An. arabiensis* mosquitoes with two subsequent blood meals. Before blood-feeding, female mosquitoes were mated with male mosquitoes over four days (1:1), after which the male mosquitoes were removed from the cages. The mated female mosquitoes were 5-days-old at the first feeding, and 9-days-old at the second feeding. Histamine present in the mosquito's salivary glands did not induce an itch-associated response in mice, thus the mice did not experience discomfort after blood feeding (Kitagawa, 1997). However, to prevent discomfort during feeding, the mice were anaesthetised with a mix of Xylazine and Anaket (0.02mL-0.03mL). Once anaesthetised, each mouse was placed on top of their respective mosquito cage (BugDorm, 4M1515).

Each cage contained a total of 100-mated female mosquitoes, which were starved 12-hours prior to feeding. The mosquitoes were allowed to feed for a total of 35-minutes, after which the mice were removed from the top of each cage. The mosquitoes had a feeding success of

approximately 80% per feeding, which was a 66.3% overall feeding success per treatment (2 feedings, 3 replicates, n=199). Mosquitoes that did not take a blood meal were subsequently removed from the study after feeding. ELISA (enzyme-linked immunosorbent assay) was performed on three blood-fed mosquitoes, to determine whether antibodies, from the vaccinated mice, were ingested by the mosquitoes, and if they could be detected. Three engorged mosquitoes were selected from each treatment, 24-hours after the final feeding (3 mosquitoes per replicate, 3 replicates, n=9 mosquitoes per treatment). Selected mosquitoes were transferred into separate microcentrifuge tubes and stored at -80ºC.

Blood samples were also collected from the saphenous vein of the mice, 24-hours before each immunisation and blood-feeding, to determine the antibody titres elicited within the mice. The mice were warmed, before blood collection, by placing a lamp over the cage for 5-minutes. To collect blood, the mice were placed in a restraining tube, so that the head of the mouse was covered, leaving the hind legs of the mouse exposed (Hoff, 2000). The skin between the tail and the thigh was grasped to obtain the saphenous vein. Hair was removed from the area using clippers, after which petroleum jelly will be applied to the area to prevent the migration of blood into the surrounding hair. A tourniquet was placed around the leg, above the knee, to allow for puncturing of the vein.

The vein was punctured using a 27.5-gauge needle, where drops of blood were collected. A total of 150µL of blood was collected from each mouse, per collection, and dispensed into separate collection tubes. The blood was incubated at room temperature for 30-minutes, to allow for coagulation. Centrifugation was conducted to separate serum from the red blood cells (1,500 x *g*, 10-minutes, 4°C). Serum was transferred to new microcentrifuge tubes, and stored at -80ºC, until an indirect ELISA was performed on the samples. The identity of each treatment was revealed once the last blood sample was completed.

An indirect ELISA was used to assess the antibody titre within each mouse (Figure 5.3). The immobilised recombinant proteins were paired to the respective immunogen for determination of the respective antibody titres. To do this, each recombinant protein was used to coat the respective 96-well microplate. The recombinant protein (0.1µg) was diluted in 100µL tris buffered saline (25 mM Tris HCl, 150 mM NaCl, 2 mM KCl) (TBS), and immobilised to the microplate by overnight incubation (4°C). After overnight incubation, the microplate was blocked with Blocker™ BLOTTO (ThermoFisher Scientific, 37530) (2-hours, 37°C). The

blocking solution was aspirated off the microplate. For the quantification of mouse antibodies, the mouse sera samples or the TBS control samples were diluted in blocking solution (1:20, v/v), and hybridised to the microplate (2-hours, 37°C). For the quantification of antibodies taken up by the mosquitoes, the fed mosquitoes were homogenized in 200µL TBS-Tween 20 (TBS-T), and centrifuged to collect the supernatant (3-minutes, 16,000 × *g*, 4°C). The supernatant was subsequently hybridised to the microplate (2-hours, 37°C). After hybridisation, the microplate was washed 3-times with TBS-T. Goat anti-mouse IgG antibody, conjugated with horseradish peroxidase (HRP) (Agilent, P0447), was diluted in blocking buffer (1:3,000). The diluted secondary antibody (200µL) was bound to the primary antibody through a 2-hour incubation (37°C). Unbound secondary antibody was aspirated off the microplate, which was subsequently washed 3-times with TBS-T. SigmaFast™ OPD enzyme-substrate (Sigma-Aldrich, N1891) was added to the microplate (200µL). After a 30-minute incubation period, the microplate was analysed at 450nm using the multi-well plate reader. The antibody titres were expressed as $OD_{450nm}$ ($OD_{\text{mouse serum or mosquito sample}} - OD_{\text{TBS control}}$), where a sample consisting of TBS was used as a background control. The antibody titres were compared between the treated and control groups, using a Student's t-test ($p<0.05$).

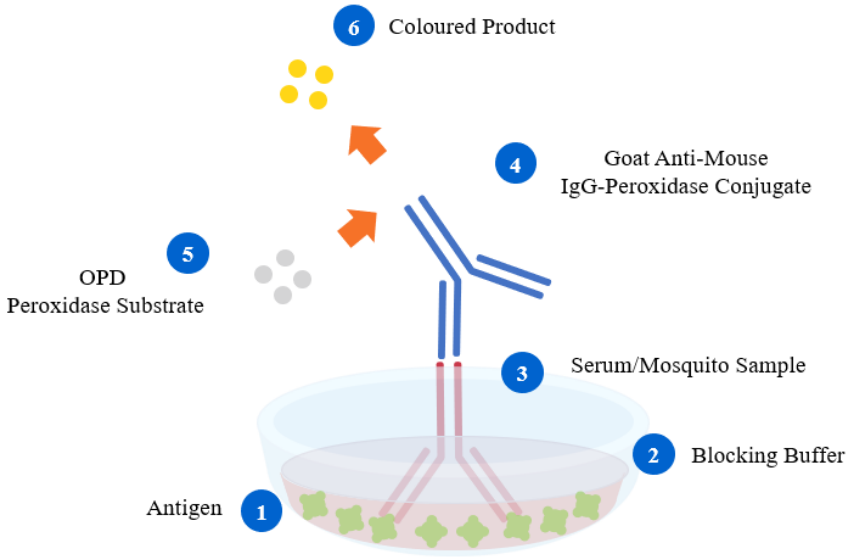


**Figure 5.3** An overview of the indirect ELISA overview used to assess antibody titres. 1: The recombinant protein was immobilised to the microplate. 2: The microplate was blocked with a blocking solution. 3: Samples were hybridised to the microplate. 4: Goat anti-mouse IgG antibody, conjugated with horseradish peroxidase, was bound to the samples. 5: OPD peroxidase substrate was added to the microplate. 6: A coloured product was formed if the antibodies were present in the sample.

### 5.2.3 Evaluation of the effect of ingested anti-Akirin antibodies on *An. arabiensis* lifetable parameters

To determine if the ingestion of anti-Akirin antibodies influenced *An. arabiensis* longevity, the rate of survival was monitored until 100% mortality was reached for all four treatments. A Kaplan-Meier survival curve was constructed using Statistix 10. The Log-rank test was used to determine whether there was a statistically significant difference in vector longevity between the treatments ($p<0.05$). See chapter 2 for detailed method.

Simultaneously, vector fecundity and fertility were also analysed. Two days after receiving the second blood meal, filter paper egg plates were placed at the bottom of each cage. The eggs laid in each plate were counted daily, over five days. Once the eggs were counted, they were placed into separate plastic containers, filled with 500mL of $dH_2O$ (L:226mm, x W:166mm x H:10mm). The hatchlings were counted over 14-days. Vector fecundity was determined by calculating the mean number of eggs laid per female mosquito, and vector fertility was determined by calculating the mean percentage of hatchlings per female mosquito. A one-way ANOVA test, and a Tukey-HSD all pairwise comparison test, was used to compare the fecundity and fertility between the treatments ($p<0.05$).

Vaccine efficiency was used to interpret the overall effect of each antigen on the *An. arabiensis* population tested. This parameter cannot be compared between vector species, as their developmental processes may differ (Moreno-Cid *et al.*, 2013). However, vaccine efficiency is a good method to compare the effect of different antigens. Efficiency (E) was calculated considering the effect of vaccination on the reduction of *An. arabiensis* fecundity (Fc), fertility (Fr) and longevity (L) as E (%) = 100[(1-L/100)(1-Fc/100)(1-Fr/100)] (Almazán *et al.*, 2003).

## 5.3 Results

### 5.3.1 Formulation of recombinant Akirin vaccines

RNA was successfully extracted from the female *An. arabiensis* to formulate a recombinant Akirin$^{arabiensis}$ vaccine. When quantified, a mean nucleic acid yield of 15µg of RNA was isolated per sample. The 260/280 absorbance ratio of the RNA samples was approximately 2.0, which was characteristic of pure RNA. The lack of smearing observed on the RNA native gel revealed that minimal RNA degradation took place during the isolation process (Figure 5.4). The two fragments observed on the gel were indicative of good quality insect RNA. The fragment at 2,000bp, was distinctive of the 18S and 28S rRNA fragments (Ishikawa and Newburgh, 1972; Winnebeck *et al*., 2010), while the fragment observed at 156bp was typical of the 5.8S rRNA.

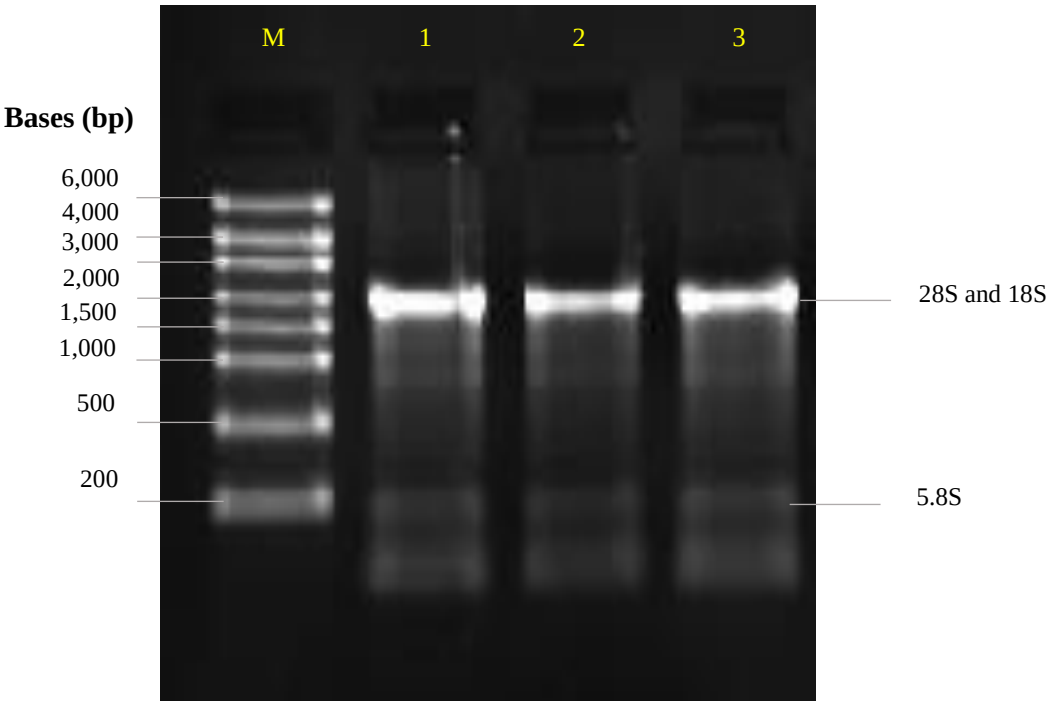


**Figure 5.4** RNA isolated from female *An. arabiensis* mosquitoes, which have been electrophoresed on a non-denaturing 1% agarose gel in TBE (70V, 30-minutes). M: RiboRuler™ high range RNA ladder (ThermoFisher Scientific, SM1823). Lanes 1 to 3: RNA isolated from female *An. arabiensis* mosquitoes.

An Akirin PCR product was successfully amplified from the RNA which was isolated from *An. arabiensis*, by reverse-transcription PCR (in one-step). After purification, the nucleotide concentration of the PCR product was quantified. A nucleotide yield total of 400ng of PCR product was amplified from the isolated RNA sample. The 260/280 absorbance ratio of the PCR product was approximately 1.8, which was characteristic of pure DNA. A single amplicon was observed on the gel electrophoresis (Figure 5.5), at approximately 613bp, which was indicative of Akirin. The absence of smearing observed on the gel was characteristic of good quality DNA. When sequenced, the Akirin RT-PCR product had an identity of 100% to the reference sequence. The Akirin RT-PCR product was subsequently cloned into the pET101/D -TOPO® vector.

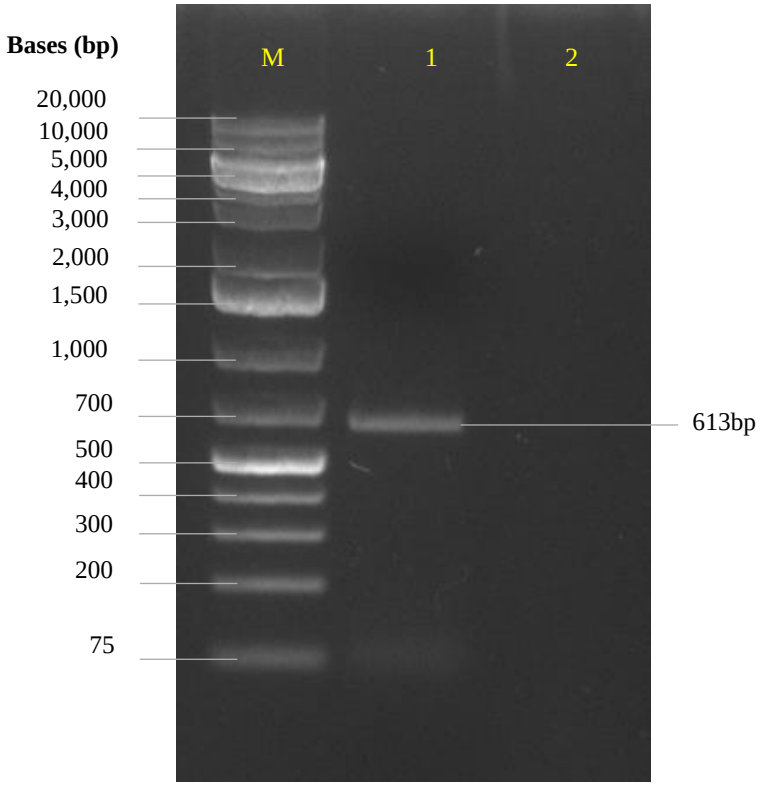


**Figure 5.5** Akirin reverse-transcription PCR product amplified in one-step, using total RNA from adult *An. arabiensis* mosquitoes, which was electrophoresed on a 1% agarose gel in TAE (100V, 120mA), M: O'GeneRuler™ 1 kb plus DNA ladder (ThermoFisher Scientific, SM1331). Lane 1: Akirin PCR product after purification. Lane 2: no template control.

After the purified RT-PCR product was cloned into the pET101/D -TOPO® vector, the vector was transformed into One Shot® TOP10 chemically competent *E. coli* cells. Transformed colonies were analysed for the Akirin insert by performing PCR amplification on selected colonies, using the Akirin forward and reverse primers. When quantified, a total of 1.25µg of PCR product was amplified per transformant. The PCR product had a 260/280 absorbance ratio of 1.8, which was characteristic of pure DNA. A single amplicon was observed at approximately 613bp (Figure 5.6), which was characteristic of Akirin. When sequenced, the PCR product had an identity of 100% to the reference sequence. The plasmid was isolated from the chemically competent *E. coli* cells, and transformed into recombinant *E. coli* BL21 Star™(DE3) One Shot® cells, to successfully express the recombinant protein, using the *E. coli* expression system.

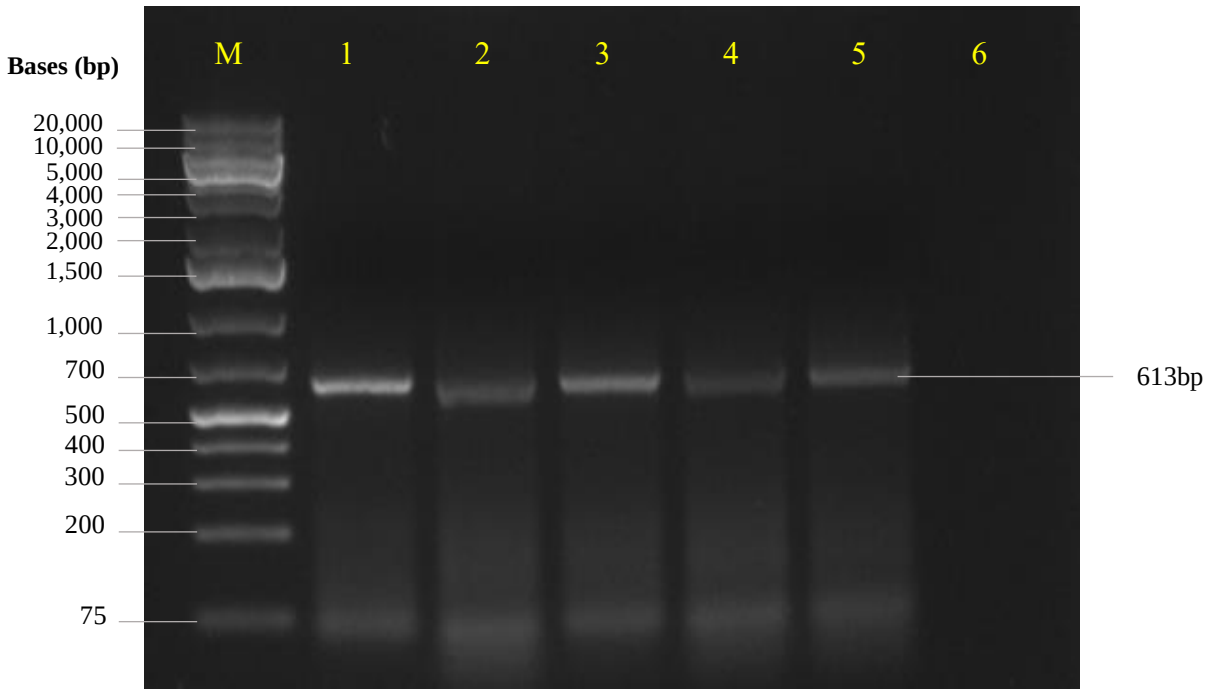


**Figure 5.6** PCR products amplified from transformants selected off LB/ampicillin agar plates, which were electrophoresed on a 1% agarose gel in TAE (100V, 75-minutes). M: O'GeneRuler™ 1 kb plus DNA ladder (ThermoFisher Scientific, SM1331). Lane 1-5: transformants containing the Akirin insert ligated within the pET101/D -TOPO® vector. Lane 6: no template control.

The transformed cells and glycerol stocks were used to successfully express the respective recombinant proteins using the *E. coli* expression system. This was attested when the eluted fractions were analysed on the SDS-PAGE gel for the respective proteins. Due to the highly conserved nature of Akirin/Subolesin, all three recombinant proteins yielded similar results, and thus only the results from Akirin$^{albopictus}$ were displayed below (Figure 5.7). The recombinant proteins were observed at 30kDa on the SDS-PAGE gel (A). The other protein bands observed in the sample was attributed to aggregated or degraded products of the recombinant antigen, as well as *E. coli* contamination proteins (Contreras *et al.*, 2017). These contaminants, however, do not affect the efficacy of the recombinant vaccines (Moreno-Cid *et al.*, 2013; Contreras *et al.*, 2017). The SDS-PAGE gel was used for Western blot analysis (B). When quantified, it was determined that 0.66mg of recombinant Akirin$^{arabiensis}$, 0.45mg of recombinant Akirin$^{albopictus}$, and 0.50mg of recombinant Q38 were expressed in total.

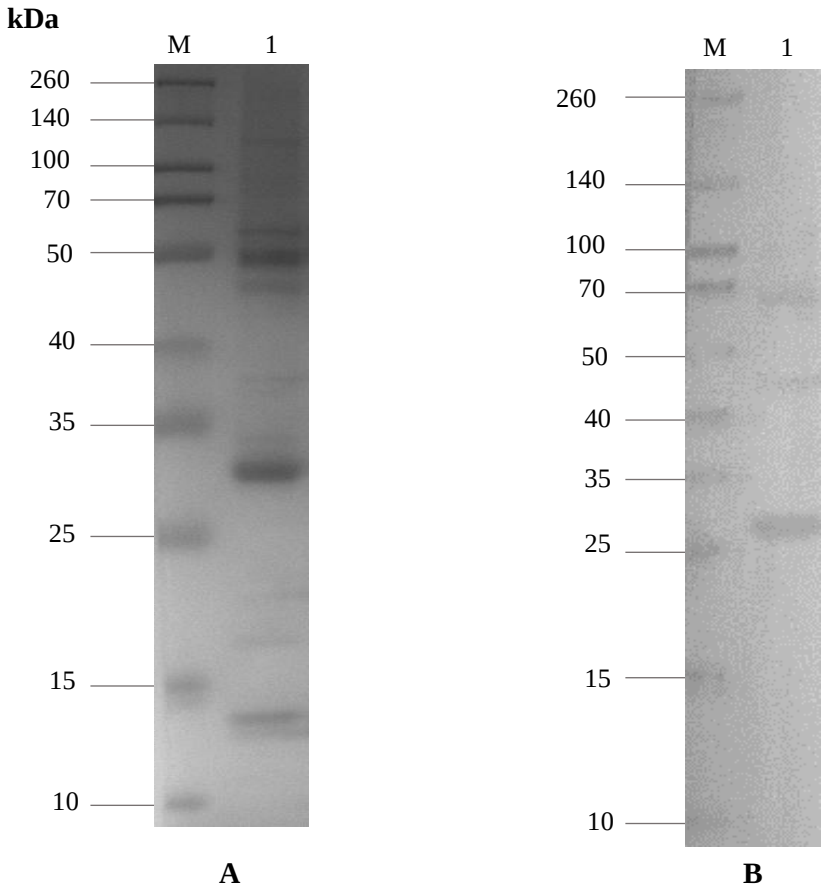


**Figure 5.7** The purified HIS-tag expressed Akirin$^{albopictus}$ recombinant protein electrophoresed on a 12% SDS-PAGE precast gel (180V, 180mA) (A), and used for western blot analysis (B). M: Spectra™ multicolor broad range protein ladder (ThermoFisher Scientific, 26634). Lane 1: eluted fraction containing the respective recombinant protein.

### 5.3.2 Performance of recombinant Akirin immunisation on balb/c mice and assessment of the antibody titres

The recombinant proteins were emulsified with an adjuvant to formulate recombinant vaccines. The mice were vaccinated intraperitoneally, with three doses of vaccine, which were administered 2-weeks apart. The antibody titres increased 10.4-fold (91%) after the first dose of the vaccine was administered (Figure 5.8). Thereafter, the antibody titres increased an additional 0.5-fold (34%) after the second dose was administered, and 0.1-fold (6%) after the third dose was administered. The antibody titres of the mice that were vaccinated with recombinant protein were significantly higher than that of the control mice. The antibody titres of the mice vaccinated with recombinant Q38 were overall 0.7-fold higher than the antibody titres of the control mice ($p$=0.04). Similarly, the antibody titres of the mice vaccinated with recombinant Akirin$^{albopictus}$ were overall 0.9-fold higher than the control mice ($p$=0.03), and the antibody titres of the mice vaccinated with recombinant Akirin$^{arabiensis}$ were overall 0.5-fold higher than the control mice ($p$=0.03).

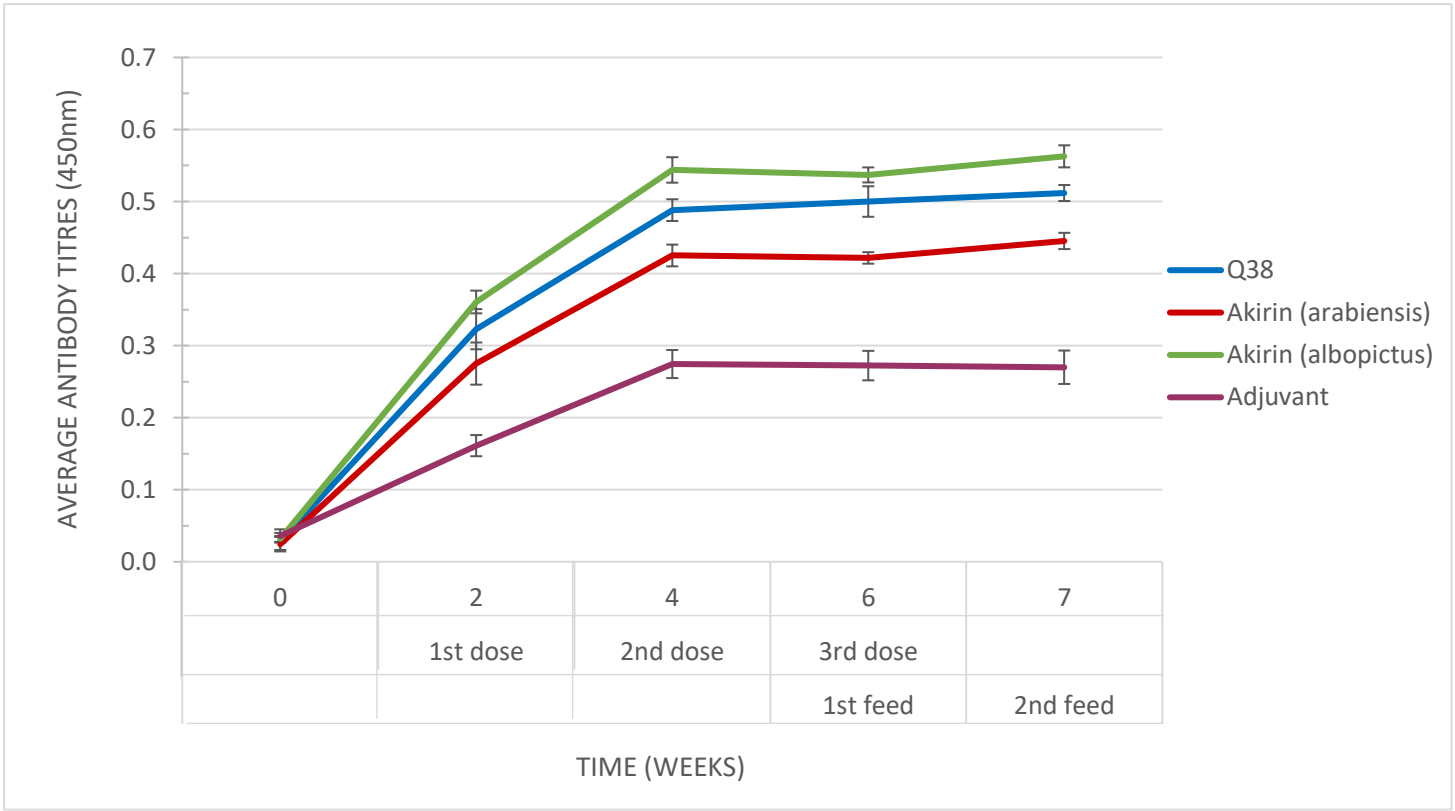


**Figure 5.8** A comparison of the average antibody titres between the mice treated with recombinant Q38 (blue), recombinant Akirin$^{arabiensis}$ (red), recombinant Akirin$^{albopictus}$ (green), and adjuvant control (purple), assessed 2 weeks after each vaccine dose was administered and before each blood meal, at an optical density of 450nm. The error bars on the graph are indicative of the S.E. between the treatment replicates.

### 5.3.3 Evaluation of the effect of ingested anti-Akirin antibodies on *An. arabiensis* lifetable parameters

The antibody titres of the females that ingested blood from mice vaccinated with recombinant protein were significantly higher than the antibody titres of mosquitoes that ingested blood from the control mice. Females that ingested anti-Q38 antibodies had an antibody titre which was 1.5-fold (61%) higher than the females that ingested blood from the control mice ($p$=0.03). *Anopheles arabiensis* which ingested anti-Akirin$^{arabiensis}$ antibodies had an antibody titre which was 2.6-fold (72%) higher than the females that ingested blood from the control mice ($p$<0.01), and the females which ingested anti-Akirin$^{albopictus}$ antibodies had an antibody titre that was 8.5-fold (90%) higher than the mosquitoes which had ingested blood from the control mice ($p$<0.01). The engorged female *An. arabiensis* mosquitoes therefore, successfully ingested antibodies from the vaccinated mice.

Vector longevity was significantly reduced in *An. arabiensis*, which ingested blood from mice vaccinated with recombinant proteins, when compared to the females that ingested blood from the control mice (Log-rank: $\chi^2$ = 84, $p$<0.01, DF=3). *Anopheles arabiensis*, which ingested blood from the control mice, had a mean survival time of 21-days, while the females that ingested anti-Q38, anti- Akirin$^{albopictus}$, and anti- Akirin$^{arabiensis}$ antibodies had a mean survival time of 16-days, 14-days and 13-days respectively. This was a 24%, 33%, and 38% reduction in mean survival time when compared to the control treatment (Table 5.2). This is a significant finding as *Anopheles* mosquitoes can transmit malaria sporozoites 14-days post-gametocyte infection.

Vector fecundity (one-way ANOVA: $p$<0.01, F=11.7, DF=3) and fertility (one-way ANOVA: $p$<0.01, F=68.9, DF=3) were also significantly reduced in *An. arabiensis* which had ingested antibodies from mice vaccinated with the recombinant proteins, when compared to the control treatment. The ingestion of anti-Akirin$^{albopictus}$ antibodies reduced vector fecundity by 1.7-fold (42%) and vector fertility by 23%. The ingestion of anti-Akirin$^{arabiensis}$ antibodies reduced vector fecundity by 2.0-fold (50%), and vector fertility by 20%. The ingestion of anti-Q38 antibodies reduced vector fecundity by 2.12-fold (53%), and vector fertility by 10%. Overall, the recombinant Q38 antigen had a vaccine efficacy of 67.8% on the targeted *An. arabiensis* vector population, while the recombinant Akirin$^{albopictus}$ antigen and the recombinant Akirin$^{arabiensis}$ antigen had a vaccine efficacy of 72.4% and 73.2% respectively.

**Table 5.2** A comparison of the lifetable parameters between the *An. arabiensis* mosquitoes which ingested blood from the control mice, and the *An. arabiensis* mosquitoes that ingested blood from mice that were vaccinated with recombinant Q38, recombinant Akirin$^{albopictus,}$ and Akirin$^{arabiensis}$ proteins.

| **Treatment** | **Mean survival time (days) ±SD (95% CI); N** | **Mean number of eggs laid per female ±SD (95% CI); N** | **Mean % of egg hatched ±SD (95% CI); N** | **Vaccine efficacy %** |
|---|---|---|---|---|
| Q38 | 16 ±1.8 (15.8-15.2); 190 [b] | 31 ±8.1 (34.0-28.4); 190 [b] | 82 ±1.7 (82.4-81.6); 190 [b] | 67.8 [a] |
| Akirin$^{albopictus}$ | 14 ± 0.6 (14.2-14.1); 190 [b] | 38 ±8.9 (40.4-34.7); 190 [b] | 70 ±1.0 (70.2-69.8); 190 [c] | 72.4 [a] |
| Akirin$^{arabiensis}$ | 13 ± 1.6 (12.9-12.4); 190 [b] | 33 ±11.2 (36.9-29.3); 190 [b] | 73 ±1.7 (73.4-72.6); 190 [c] | 73.2 [a] |
| Control | 21 ± 1.0 (20.6-20.4); 190 [a] | 66 ±4.1 (67.4-65.5); 190 [a] | 91 ±0.6 (90.8-90.5); 190 [a] | - |

*Results are shown for each vaccine trial (n=3), with the average standard deviation (±S.D.) and total sample size (N). Data was analysed using the Log-rank test and a one-way ANOVA test ($p < 0.05$). Letters "a-c" were used to represent statistical differences between various groups when conducting the Tukey-HSD test.

## 5.4 Discussion

The significant reduction in vector survival and reproductive capacities characterised by the knockdown of *akirin* expression in *An. arabiensis* (Letinić *et al.*, 2020), made Akirin a potential antigen for vaccine development against untargeted *An. arabiensis* vector populations. Thus, to determine whether Akirin could represent a novel target for the control of *An. arabiensis*, the efficacy of three recombinant vaccines were evaluated, namely recombinant Akirin$^{arabiensis}$, recombinant Akirin$^{albopictus}$, and recombinant Q38. Antibodies against the recombinant proteins were effectively elicited in vaccinated mice, and were successfully ingested by engorged *An. arabiensis,* after which vector lifetable parameters, such as fertility, fecundity, and longevity, were subsequently assessed.

Vector longevity was significantly reduced when anti- Akirin$^{arabiensis}$ antibodies, anti-Akirin$^{albopictus}$ antibodies, and anti-Q38 antibodies were ingested. Although not completely understood, it is thought that the ingested antibodies cross the cell membrane and interact with the target protein in the cytoplasm (de la Fuente *et al.*, 2011; de la Fuente *et al.*, 2013). This would prevent Akirin from translocation to the nucleus, which would disrupt the biological functions of Akirin within *An. arabiensis.*

When ingesting a blood meal, the mosquito's midgut bacterial levels increase rapidly (Pumpuni *et al.*, 1996). This causes an increase in bacterial elicitors, such as PGN, which activates the IMD pathway (Choe *et al.*, 2002; Kumar *et al.*, 2010). Activation of the IMD pathway causes DREDD-mediated cleavage of Relish (Ertürk-Hasdemir *et al.*, 2009). Relish translocates into the nucleus and binds to a complex formed by Akirin and BAP60 (Dushay *et al.*, 1996; Sutterwala and Flavell, 2008). This allows for the gene expression of several antimicrobial peptide-coding genes, which reduce the number of microbiotas in the midgut back to the basal level (Dong *et al.*, 2009; Meister *et al.*, 2009; Bonnay *et al.*, 2014). It is possible that targeting Akirin disrupts the bacterial homeostasis and weakens the innate immune defence required for survival (Ferrandon *et al.*, 2007; Goto *et al.*, 2008; Nowak *et al.*, 2012; Mukherji *et al.*, 2013; Debalke *et al.*, 2019).

As previously mentioned, vector longevity is a critical factor concerning malaria transmission. To transmit malaria, a female *Anopheles* mosquito must ingest a blood meal infected with gametocytes. The gametocytes must encounter the midgut epithelium, undergo fertilization,

develop into motile ookinetes, transverse across the midgut epithelium, develop into oocysts, produce haploid sporozoites, and migrate through the mosquito haemolymph to the salivary glands to complete the sporogonic cycle (Sinden, 1984). It takes approximately 14-days to complete the sporogonic cycle. Once complete, the sporozoites are subsequently injected into the next vertebrate host when taking a successive blood meal (Levashina, 2004; Vlachou *et al.,* 2006). This allows for the transmission of malaria. Eliminating a third of the vector population prior to the completion of this incubation period could significantly affect the transmission of malaria.

Additionally, both vector fecundity and vector fertility were significantly reduced when anti-Akirin$^{arabiensis}$ antibodies, anti- Akirin$^{albopictus}$ antibodies, and anti-Q38 antibodies were ingested by *An. arabiensis*. It has been postulated that the movement of antibodies to reproductive tissues interferes with oogenesis, as the synthesis of vitellogenin by the fat body, as well as the uptake of vitellogenin from the haemolymph, may be inhibited (Suneja *et al.,* 2003). Alternatively, it has been suggested that the ingested mosquito antibodies may aggravate the gut, which in turn reduces the availability of nutrients, thus the content of some of the developing follicles may be reabsorbed (Suneja *et al.,* 2003). Akirin is, however, also known to play a critical role in processes unrelated to immunity, such as tissue development, embryogenesis, and reproduction (Goto *et al.,* 2008; Canales *et al.,* 2009). Reducing the number of eggs laid by half, as well as the amount of viable progeny by a fifth, could reduce the *An. arabiensis* population.

Mosquitoes that have taken an infectious blood meal usually take three to four additional blood meals before completing the sporogonic period (Debalke *et al.,* 2019). This would ensure repeated ingestion of anti-mosquito antibodies, ultimately reducing the lifespan of the vector, as well as reproductive fitness. Reducing *An. arabiensis* survivorship as well as reproductive fitness would ultimately cause a reduction in vector population abundance. A reduction in vector abundance would have a significant impact on malaria transmission.

When comparing the efficacy between the three recombinant vaccines on *An. arabiensis,* the difference was negligible. However, the chimeric nature of Q38 makes this antigen an especially valuable multi-target arthropod vaccine. The efficacy of Q38 has been previously tested on *Ixodes ricinus* and *Dermacentor reticulastus* (ticks), as well as *Phlebotomus perniciosus* (sandflies) (Moreno-Cid *et al.,* 2013; Contreras and de la Fuente, 2016). The

ingestion of anti-Q38 antibodies significantly reduced *I. ricinus,* longevity by 4.25-fold as well, as moulting by 38%. The ingestion of anti-Q38 antibodies by *D. reticulastus* significantly reduced the mass of the vector by 25%, as well as moulting by 15% (Contreras and de la Fuente, 2016). When anti-Q38 antibodies were ingested by *P. perniciosus,* oviposition was reduced by 16–26% (Moreno-Cid *et al.,* 2013). This displays the need to investigate the impact of dual vaccines using various vectors.

Additionally, the reduction in *An. arabiensis* longevity after the ingestion of anti-Akirin$^{arabiensis}$ antibodies and anti-Akirin$^{albopictus}$ antibodies was an interesting observation, as previous recombinant Akirin$^{albopictus}$ immunisation experiments affected *Ae. aegypti*, *Ae. albopictus,* and *An. coluzzii* oviposition, but not vector longevity (Moreno-Cid *et al.,* 2010; da Costa *et al.,* 2014). These differences are attributed to the physiological difference between the various genera and species, such as the amount of protein ingested by each vector species, as well as the biological activities of the mosquitoes, which are influenced by sensitivity to proteases within the midgut (Chugh *et al*., 2011).

Another possible reason for the variability of these results could be due to the systems used to express these recombinant vaccines. In previous immunisation studies, the *Pichia pastoris* expression system was used to express recombinant Akirin$^{albopictus}$ and recombinant Q38 antigens (Moreno-Cid *et al.,* 2010; Moreno-Cid *et al.*, 2013; da Costa *et al.,* 2014), while this investigation used the *E. coli* expression system. Moving forward, mosquito vaccine research could be standardised to rule out variations due to the techniques used to express these recombinant vaccines, similar to that proposed for tick vaccines (Schetters *et al*., 2016). The standardisation of mosquito vaccine parameters would need to include the expression system used, the type of adjuvant used, the experimental procedures followed to determine the antibody titres and vaccine efficacy, the sample size used to make these comparisons, as well the age of the experimental animals used within the study. This would allow for a more accurate comparison of recombinant antigen results between different research groups.

Nonetheless, challenging various blood hosts with whole protein or antigenic peptides may provide a valuable intervention against several ectoparasites including the untargeted exophagic *An. arabiensis* populations. This could be a valuable intervention for the control of *An. arabiensis,* as the human malaria parasites are unable to amplify within these reservoir hosts. The conserved structure and function of Akirin throughout the metazoan world (Almazán

*et al.,* 2005) does, however, raise the question of safety when vaccinating vertebrates due to autoimmunity (Canales *et al.*, 2009; de la Fuente *et al.,* 2011).

As in previous experiments performed on various blood hosts (Canales *et al.*, 2009; Moreno-Cid *et al.,* 2010; Merino *et al.,* 2011; de la Fuente *et al.,* 2013; Contreras and de la Fuente, 2016), physiological and pathological alterations were not observed in the vaccinated mice. This suggests that as expected, the antibody response was directed against non-self-epitopes, thus reducing the possibility of detrimental effects to the vaccinated host (Canales *et al.*, 2009). It is possible that the intracellular nature of the Akirin antigen prohibited the anti-Akirin antibodies entering mammalian cells of the mice (de la Fuente *et al.,* 2011), however further research needs to be done to confirm this. Additionally, effective immunisation of various hosts, for the control of numerous vector infestations, displays the low risk of inducing an autoimmune response in vertebrates (Canales *et al.*, 2009; Moreno-Cid *et al.*, 2010; Merino *et al.,* 2011; de la Fuente *et al.,* 2013; Contreras and de la Fuente, 2016).

Blood hosts vaccinated with recombinant Akirin$^{arabiensis}$, recombinant Akirin$^{albopictus,}$ or recombinant Q38 vaccines could, therefore, be used to target the *An. arabiensis* exophagic vector population. This vector control strategy could also impact secondary malaria vectors, or vectors of other diseases, if similar detrimental effects are caused within these vectors. Further studies, however, still need to be performed to determine if agricultural or domestic animals, vaccinated with recombinant Akirin vaccines, are able to elicit a similar response against *An. arabiensis* to those observed when recombinant Akirin vaccines were evaluated using balb/c mice. Additional research will also need to be conducted to determine the efficacy of these recombinant vaccines, due to the difference in feeding preferences of the wild *An. arabiensis* population. Furthermore, the half-life of these antibodies will need to be assessed within the reservoir hosts, to determine how often booster vaccines will need to be administered to the agricultural or domestic animals.

Therefore, additional studies will need to be performed to determine if agricultural or domestic animals can be vaccinated with recombinant Akirin antigens, to aid in the suppression of the *An. arabiensis* exophagic vector population. By reducing *An. arabiensis* survivorship as well as reproductive fitness, a reduction in vector population abundance would ultimately occur. A reduction in vector abundance would have a significant impact on malaria transmission, especially in countries with low-level seasonal malaria transmission, such as South Africa.

## CHAPTER 6: DISCUSSION

The pleiotropic nature of Akirin made targeting this nuclear transcription co-factor a viable option for *An. arabiensis* vector control. A better understanding of Akirin's function in *An. arabiensis* fecundity, fertility, and longevity was determined by performing CRISPRi, RNAi, and recombinant Akirin immunisation. Each technique was used to target Akirin at a different level of gene regulation. CRISPR interference was used to target Akirin transcription, while RNAi was used to target Akirin translation, and recombinant protein immunisation was used to target Akirin translocation. At each level of gene regulation, a significant reduction in vector survivorship and/or reproductive fitness resulted when Akirin was targeted. While these results cannot be directly compared to each other, they do show that Akirin can be targeted in *An. arabiensis*, using several different techniques.

When Akirin was targeted using Akirin-specific sgRNA and dCas9, the decrease of relative *akirin* expression in *An. arabiensis* resulted in an 18% decrease in vector longevity, when the mean survival time was compared to the control treatments. When Akirin was targeted in *An. arabiensis* using Akirin-specific siRNA or Akirin-specific dsRNA there was a 29-46% reduction in mean survival time, 17-25% decrease in vector fecundity, and 19-29% decrease in vector fertility in relation to the control treatments. When Akirin was targeted using recombinant Akirin$^{arabiensis}$, recombinant Akirin$^{albopictus}$, or recombinant Q38 protein vaccines, a 24-38% reduction in mean survival time, 42-53% decrease in vector fecundity, and 10-23% decrease in vector fertility occurred in relation to the control treatment. A reduction in vector survival, as well as reproduction, would have a significant impact on residual malaria transmission, in countries like South Africa, due to a reduction in vector abundance (Killeen, 2014; Killeen *et al.,* 2016).

From previous studies, it is known that Akirin acts as a nuclear transcription co-factor that regulates NF-κB activity (Goto *et al.,* 2008). This subsequently causes Akirin to play a crucial role in innate immunity, which is imperative for vector survival. Akirin is also functionally important for other molecular pathways, including those required for tissue development and embryogenesis (Almazán *et al.,* 2005; Goto *et al.,* 2008; Macqueen and Johnston, 2009; de la Fuente *et al.,* 2010). However, the Akirin interactome has not yet been fully characterised (Artigas-Jerónimo *et al.,* 2017). Transcriptomic studies could therefore be conducted to determine how Akirin-specific sgRNA, Akirin-specific dsRNA, or anti-Akirin antibodies

affect *An. arabiensis* on a molecular level, when compared to untreated *An. arabiensis* females. This could provide a better understanding of Akirin's function in this malaria vector.

Additional phenotypic evaluations could also be conducted to determine whether the effects of *akirin* knockdown persist in the subsequent generation of *An. arabiensis*, post-hatching. Previously, the generation of Akirin null mutant *Drosophil*a resulted in several lethal phenotypes characteristic of defective muscle patterning, including improper muscle attachment, muscle duplication, and muscle absence (Goto *et al.*, 2008). Furthermore, the downregulation of *subolesin* expression caused several deleterious effects in engorged ticks. These included the failure of nymph metamorphosis, as well as tissue damage in various tick species (Almazán *et al.*, 2005; de la Fuente *et al.*, 2010). Analysing the effects of *akirin* knockdown in the *An. arabiensis* progeny, post-hatching, would further aid in determining whether targeting Akirin is a viable option for *An. arabiensis* vector control.

It would also be beneficial to evaluate the effect of recombinant Akirin antibodies on *P. falciparum* infection, as well as the effect of infectivity in *An. arabiensis*. Previous studies conducted in *An. coluzzii* looked at the role of Akirin on *P. berghei* parasite infection (da Costa *et al.*, 2014). The ingestion of Akirin$^{albopictus}$ antibodies reduced *P. berghei* infection in *An. coluzzii*, which suggested that recombinant Akirin vaccines could also aid in suppressing malaria transmission, by reducing parasite infection levels in vector mosquito species. *Plasmodium berghei* is, however, the rodent malaria parasite, while malaria transmission in humans is caused by *P. falciparum.* In a malaria vector, the Toll pathway is more effective in the defence against *P. berghei*, while the IMD pathway has emerged as the most effective pathway in the defence against the human malaria parasite *P. falciparum* (Mitri *et al.*, 2009). Since Akirin is a crucial regulator of the IMD pathway, it would be beneficial to evaluate the effect of recombinant Akirin antibodies on *P. falciparum* infection. This would also determine whether targeting Akirin could also aid in suppressing malaria transmission, by reducing parasite infection levels in *An. arabiensis*.

Although further research is needed to fully characterise the biological role of Akirin in *An. arabiensis*, the function of Akirin in vector fecundity, fertility, and longevity has made this nuclear transcription co-factor a viable option for the control of *An. arabiensis*. However, to determine if targeting Akirin could serve as an additional control intervention in the field, similar deleterious phenotypes to those observed in a laboratory environment would need to be

achieved in wild populations of *An. arabiensis*. Several possible applications have been mentioned in the previous chapters, yet each of the applications mentioned has its limitations.

As previously mentioned, zooprophylaxis could be implemented, in combination with other vector control strategies, to aid in targeting wild *An. arabiensis* exophagic vector populations. Agricultural or domestic animals, which are not reservoir hosts of human malaria, could be used to divert *An. arabiensis* away from human hosts (Habtewold *et al.,* 2004; Mahande *et al.,* 2007). The results obtained when Akirin was targeted using recombinant vaccines, were the first showing that blood hosts vaccinated with recombinant Akirin vaccines could aid in the suppression of *An. arabiensis* vector populations, through the reduction of vector survivorship and reproductive capacities. Therefore, blood hosts vaccinated with recombinant Akirin$^{arabiensis}$, recombinant Akirin$^{albopictus,}$ or recombinant Q38 vaccines could be used to target the *An. arabiensis* exophagic vector population. This vector control strategy could also impact secondary malaria vectors, or vectors of other diseases, if similar detrimental effects are caused within these vectors.

Further studies, however, still need to be performed to determine if agricultural or domestic animals, vaccinated with recombinant Akirin vaccines, are able to elicit a similar response against *An. arabiensis* to those observed when recombinant Akirin vaccines were evaluated using balb/c mice. Additional research will also need to be conducted to determine the efficacy of these recombinant vaccines, due to the difference in feeding preferences of the wild *An. arabiensis* population. The half-life of these antibodies will also need to be assessed within the reservoir hosts, to determine how often booster vaccines will need to be administered to the agricultural or domestic animals. Furthermore, the effect of these vaccines on human consumables such as meat/milk will need to be evaluated.

Transgenic plants could also be used as an alternative application for targeting Akirin in wild *An. arabiensis* populations. In nature, the most common sources of sugar for mosquitoes are floral-nectar, extrafloral-nectar, honeydew, tree sap, rotting or damaged fruit, and portions of damaged leaves (McCrae *et al.,* 1969; Foster, 1995). These plants could potentially be engineered to express hairpin RNAs that are functionally equivalent to that of the linear Akirin-specific dsRNA. To limit the effects of *akirin* knockdown in unintended arthropod species, RNAi triggers would need to be designed around a portion of the Akirin sequence which is unique to *An. arabiensis* (Whyard *et al.,* 2009; Goto *et al.,* 2008).

Although Akirin is highly conserved at the N-terminal and the C-terminal domains, these domains are separated by a stretch of less conserved residues (Goto *et al.,* 2008). The stretch of less conserved residues could therefore be used to design an Akirin-specific dsRNA that only targets *An. arabiensis.* The hairpin RNAs would subsequently be ingested by *An. arabiensis* when taking a sugar-meal. This would result in the downregulation of *akirin* expression in the malaria vector. However, this application has not yet been evaluated in Culicidae, and thus would need further investigation. Using transgenic plants to target Akirin in *An. arabiensis* may, however, cause public concerns with regards to human and environmental safety, due to the controversies surrounding the use of genetically modified crops (Frewer and Salter, 2002).

Alternatively, *E. coli* expression systems could be repurposed to express plasmids containing the Akirin-specific sgRNA or Akirin-specific dsRNA. These constructs could be mixed with a sucrose-meal or an artificial blood-meal, which would then be delivered to *An. arabiensis* by oral ingestion, in the form of attractive-toxic sugar baits (Qualls *et al.,* 2014; Zhu *et al.,* 2015; Ding *et al.,* 2016). Insect expression plasmids have also been shown to persist through multiple generations (Peng *et al.,* 2011). This would provide a platform to develop an efficient gene-drive system, that could be used to confer the population suppression phenotype to wild populations of *An. arabiensis.* Further research would, however, needs to be performed to evaluate the stability and feasibility of these constructs.

Another possible strategy to introgress the Akirin population suppression phenotype into wild populations of *An. arabiensis,* would be to use a CRISPR/Cas9 gene-drive system (Zhang and McCarty, 2017). The CRISPR/Cas9-based genome editing technique has previously been used to achieve permanent gene inactivation in *An. gambiae,* through CRISPR/Cas9-induced somatic gene disruption (Hammond *et al.,* 2016; Dong *et al.,* 2018). In theory, an Akirin knockout line could be crossed with a germline-Cas9 strain to create a germ-line gene-knockout line of Akirin in *An. arabiensis*. However, this would need to be tested in cage trials, where the mosquito life-table parameters and mating competitiveness can be observed in a controlled environment, using different ratios of genetically modified to unaltered individuals.

The effect of *akirin* knockout has yet to be established in *Anopheles*. Should complete inactivation of Akirin result in an immediate lethal phenotype, it would be impossible to use this technique as a once-off gene-drive tool. If successful, this line of mosquitoes could be released near the existing populations of *An. arabiensis* to introgress the life-shortening trait into the wild vector population (Peng *et al.,* 2011). This application may, however, be problematic as this would entail releasing potential malaria vectors into the wild. The dispersal of these genetically modified mosquitoes outside of the intended target zones could cause the resurgence of malaria vectors in areas that have already achieved local elimination (Macias *et al.,* 2017). This could cause political tensions with bordering countries that have not approved this application. If implemented, ongoing surveillance will need to be conducted to interpret the success of the release strategy, identify unexpected genetic mutations, and determine if a reversal of the gene-drive is necessary (Wu *et al*., 2016; Macias *et al.,* 2017).

Although further research is needed to determine whether Akirin can be targeted in wild populations of *An. arabiensis*, the reduction in vector fecundity, fertility, and longevity has made targeting Akirin a viable option for the control of *An. arabiensis*. The results presented in this study were the first to characterise the biological role of Akirin in *An. arabiensis* survival and reproduction, by performing CRISPRi and RNAi, using an optimised nano-injection protocol. They were also the first to show that blood hosts vaccinated with recombinant Akirin vaccines could aid in the suppression of *An. arabiensis* vector populations, through the reduction of vector survivorship and reproductive capacities. These results have subsequently provided a step towards the development of a novel target for *An. arabiensis* vector control.

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# APPENDICES

## APPENDIX A

Primers designed to generate the sgRNA dsDNA template.

**Table A.** Primers designed to generate the sgRNA dsDNA template for *in vitro* transcription. The forward primer contained the T7 promoter (red), the sgRNA target sequence (purple), and the obligate GG (green) sequence. The reverse primer contained the universal CRISPR reverse primer that could be used to design all sgRNA templates (GenBank: KX446948.1). The **bold** portion of each sequence is the complementary sequences, used to anneal the forward and reverse primers to generate the sgRNA template.

| Gene | Primer | Sequence |
|---|---|---|
| **Akirin** | CRISPR_F: | 5′GAAATTAATACGACTCACTATAGGCCGCTGCAACGGATGTGG**GTTTTAGAGCTAGAAATAGC**3′ |
| | CRISPR_R: | 5′AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTT**GCTATTTCTAGCTCTAAAAC**3′ |
| **Mouse-β2m** | CRISPR_F: | 5′GAAATTAATACGACTCACTATAGGTCGCTTCAGTCGTCAGCA**GTTTTAGAGCTAGAAATAGC**3′ |
| | CRISPR_R: | 5′AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTT**GCTATTTCTAGCTCTAAAAC**3′ |

# APPENDIX B

Sequences used to perform RNA-mediated interference in *An. arabiensis* female mosquitoes, using Akirin-specific siRNA, Mouse-β2m-specific siRNA, and GapDH-specific siRNA.

**Table B.** siRNA sequences used to conduct RNAi.

| Gene | Accession Number | Primer | Amplicon size (bp) | Position (bp) |
|---|---|---|---|---|
| Akirin | VectorBase: AARA009142 | 5′AUAUCACGGAGGAAAUCAAtt3′ | 19 | 991-1009 |
| | | 5′UUGAUUUCCUCCGUGAUAUtt3′ | | |
| Mouse-β2m | GenBank: NM_009735 | 5′GCCUGUAUGCUAUCCAGAAtt3′ | 19 | 101- 119 |
| | | 5′UUCUGGAUAGCAUACAGGCcg3′ | | |
| GapDH | VectorBase: AARA011366 | 5′AGAUCGGAAUUAACGGAUUtt3′ | 19 | 8-26 |
| | | 5′AAUCCGUUAAUUCCGAUCUtc3′ | | |

# APPENDIX C

**Ethics clearance certificates:**

- **Waiver BD Letinic 201803222**
  Waiver from the Animal Research Ethics Committee of the University of Witwatersrand

- **Clearance Certificate No. 2017/010/72/B**

  Animal ethics clearance from the Animal Research Ethics Committee of the University of Witwatersrand

- **Clearance Certificate No. AEC001-19**
  Animal ethics clearance from the Animal Research Ethics Committee of the National Institute for Communicable Diseases

**ANIMAL RESEARCH ETHICS COMMITTEE**
Registration number: AREC-101210-002

March 23, 2018

To whom it may concern,
*Reference :* ***Waiver BD Letinic 201803222***
Re: Waiver from the Animal Research Ethics Committee of the University of the Witwatersrand

Ms. Blazenka Danica Letinic is a PhD candidate (by upgrade) undertaking a study over the next 3 years, titled "The biological role of *Akirin* in *Anopheles arabiensis,* to evaluate its use as a tool in vector control".

The research is being conducted in the Maureen Coetzee Insectary, at the Wits Research Institute for Malaria (WRIM) of the University of the Witwatersrand (WITS). The research is being supervised by Professor Lizette Koekemoer.

This letter is to confirm that

- 16x 5 week old female balb/c mice will be used in this study (2017/010/72/B). No further animals will be used in this study. This has been approved as separate the separate AREC clearance (2017/010/72/B);
- the remained of the research being conducted on the mosquitoes and thus does not require further Animal Research Ethics Committee clearance to undertake the work and is covered under this waiver;
- the applicant has indicated : "that appropriate biosafety measures will be employed. All mosquitoes will be housed in our secured insectary and will be disposed of in an appropriate manner upon death";
- copies of this waiver and the AREC clearance certificate must be included in any submitted research report/thesis/dissertation.

Please contact me should you require further information,

Yours sincerely

GP Candy

Geoffrey Candy
Chair : Animal Ethics Research Committee
University of the Witwatersrand

*Geoffrey P Candy PhD*
*Professor, Department of Surgery, Faculty of Health Sciences,* University of the Witwatersrand, *7 York Road Parktown 2193, Johannesburg; Tel: 27-11-717-2574; Email: geoffrey.candy@wits.ac.za*

**STRICTLY CONFIDENTIAL**

**ANIMAL ETHICS SCREENING COMMITTEE (AESC)**

**CLEARANCE CERTIFICATE NO.** 2017/010/72/B

**APPLICANT:** Ms B Letinic

**SCHOOL:** Wits Institute for Malaria
**DEPARTMENT:**
**LOCATION:**

**PROJECT TITLE:** The biological role of Akirin in Anopheles arabiensis at both a phenotypic and a transcriptional level, to evaluate its use as a tool in vector control

**Number and Species**

16X 5week old female Balb/c mice

Approval was given for the use of animals for the project described above at an AESC meeting held on 2017/10/31. This approval remains valid until 2019/12/04.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

Signed: _____ (Chairperson, AESC) Date: 12 January 2018

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 10 January 2018

cc: Supervisor: N/A
Director: CAS

Works 2000/lain0015/AESCCert.wps

Version: CC 1/19 Date: Jun 2019

***NATIONAL INSTITUTE FOR COMMUNICABLE DISEASES***
***ANIMAL ETHICS COMMITTEE***


| Chairperson: Dr. Petrus Jansen van Vuren | Secretariat: Mrs Busisiwe Mogodi |
|---|---|
| NICD/NHLS Centre for Emerging Zoonotic and Parasitic Diseases | NICD/NHLS Centre for Emerging Zoonotic and Parasitic Diseases |
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## ANIMAL ETHICS CLEARANCE CERTIFICATE

CLEARANCE CERTIFICATE NUMBER: AEC001-19

APPLICANT: BLAZENKA LITINIC

DEPARTMENT/COMPANY: WITS RESEARCH INSTITUTE FOR MALARIA

PROJECT TITLE: THE BIOLOGICAL ROLE OF AKIRIN IN ANOPHELES ARABIENSIS

| SPECIES | NUMBER | TYPE OF APPLICATION |
|---|---|---|
| MICE (BALB/C) | 16 | RESEARCH |

i) Approval is hereby given for the procedure/s described in the above application.

The use of this material is subject to the National Standard SANS 10386: 2008 guidelines as used by the NICD AEC. If an application is for a routine procedure then the recommended guidelines or SOP must be followed. It is limited to the procedure/s specified in the application form and to: attached schedule of tests

SIGNED---------- DATE: 20 JUNE 2019
(Chairperson: Animal Ethics Committee)

ii) I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23(1) (C) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

SIGNED---------- DATE: 28/04/2019
(Registered Veterinarian)

# APPENDIX D

**Letinić, B.D., Kemp, A., Christian, R.N. and Koekemoer, L.L.**, 2018. Inoculation protocol for the African malaria vector, *Anopheles arabiensis,* by means of nano-injection. *African Entomology*, *26*(2), pp.422-428.

| **Author** | **Name** | **Signature** | **Date** |
|---|---|---|---|
| 1st Author | Blaženka D. Letinić |  | 14 July 2020 |
| 2nd Author | Alan Kemp |  | 14 July 2020 |
| 3rd Author | Riann N. Christian |  | 17 July 2020 |
| 4th Author | Lizette L. Koekemoer |  | 15 July 2020 |

**Comments by Primary Supervisor:**

Ms Letinić wrote the first and subsequent drafts of the paper, conducted the experimental work and performed analysis. Contribution of co-authors are detailed in the manuscript.

# Inoculation protocol for the African malaria vector, *Anopheles arabiensis*, by means of nano-injection

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Inoculation by means of injection has become a prominent bio-manipulation technique. This physical delivery system offers an advantage over other techniques by introducing precise quantities of inoculate into any life stage of an organism. However, this technique is intricate, laborious and requires extensive optimisation. Factors such as the location of injection, age of the organism, injection volume, and nutritional status of the organism prior to injection are variables that will likely differ between species. Bio-manipulation studies have been performed on the major African malaria vector mosquito species, *Anopheles gambiae s.s.*, yet they are still lacking for the closely related vector *An. arabiensis*. This study established a method of nano-injection procedure for *An. arabiensis* mosquitoes and found that the highest rate of survival was achieved when 1-day-old mosquitoes, fed on a 10 % sucrose solution prior to injection, were intra-thoracically injected using an inoculation volume of 69 nl.

**Key words**: bio-manipulation, *An. gambiae* complex, injection protocol.

## INTRODUCTION

Inoculates can be introduced into target insects through transfection and transformation. These substances can be used to affect an insect's genome, by the insertion of detectable markers, or they can be designed to affect a target insect's ability to transmit human and animal diseases, as well as its susceptibility to pesticide, or they can be used to induce sterility (Sampath & Puttaraju 2012). The inoculation of particular compounds, such as double stranded RNA (dsRNA), into target organisms is widely used to answer diverse research questions such as the biological function of gene transcripts. Inoculation can be done through a technique known as nano-injection.

The first dsRNA nano-injection studies reported in insects were conducted by Kennerdell & Carthew (1998), to demonstrate the function of the genes *frizzled* and *frizzled*-2 in the wingless pathway of *Drosophila melanogaster*. Since then, nano-injection has become a prominent bio-manipulation technique. However, this technique is intricate, laborious, and requires extensive optimisation with regards to needle length, location of inoculation, inoculation volume, and age of the organism being inoculated, as these factors may differ between organisms and between species (Sampath & Puttaraju 2012).

Sampath & Puttaraju (2012) conducted inoculation trials on *Aedes aegypti, Culex quinquefasciatus* and *An. stephensi* mosquitoes *via* the abdomen. These trials were performed on 1- to 12-day-old mosquitoes in starved, sucrose-fed and blood-fed conditions, using approximately 3 $\mu$l of phosphate-buffered saline (PBS). Adult 3-day-old mosquitoes had the highest survival rate post-inoculation when compared to the other age clusters across all three species. The same study showed that a 12-h sucrose starvation prior to inoculation increased the rate of survival when compared to mosquitoes that were sucrose- or blood-fed immediately prior to inoculation.

The nano-injection technique has also been successfully used to administer various constituents, such as dsRNA, to the major African malaria vector mosquito *An. gambiae* (Blandin *et al.* 2002; Lycett *et al.* 2006; Catteruccia & Levashina 2009; Da Costa *et al*. 2014). These nano-injection studies were conducted on 1- to 3-day-old *An. gambiae*

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mosquitoes that were injected intra-thoracically with 69 nl of inoculation solution.

The success of nano-injection experiments on *An. gambiae* provided the impetus to initiate studies using this technique on another closely related malaria vector species, *An. arabiensis*. This species is morphologically indistinguishable from *An. gambiae* (Sinka *et al.* 2010) and is a major vector of malaria in countries like South Africa (Dandalo *et al.* 2017), Eritrea (Shililu 2001), Ethiopia and Sudan (Gillies & de Meillon 1968; Gillies & Coetzee 1987). The aim of this study was to establish a nano-injection protocol for *An. arabiensis,* so as to enable future bio-manipulation studies on this species, including gene knockdown using RNAi.

## METHODS

### *Biological material*

A laboratory strain of *An. arabiensis* (MBN-Base) was used for experiments. This colony was initiated in 2002, from wild material collected in Mamfene, KwaZulu-Natal Province, South Africa (Hargreaves *et al.* 2003). This strain was reared under conditions of 80 % humidity, 25 °C, and a 12-hour day/night cycle with 45-minute dusk/dawn transitions (Hunt *et al.* 2005).

### *Nano-injection*

The effects of four parameters (injection location, mosquito feeding status, mosquito age, and injection volume) were assessed in terms of mosquito survival post-injection, in order to establish a laboratory procedure for future inoculation experiments. Each parameter was evaluated in triplicate, using 20 female adult mosquitoes per replicate. Kaplan-Meier survival curves were used to plot the survivorship of each parameter tested over a 10-day period, using a statistical program (Statistix 10). Log-rank, Wilcoxon and Tarone-Ware analysis were used to determine the statistical difference in survivorship at different time periods within the survival curves. Phosphate-buffered saline solution (pH 6.7) was used as the injection solution, as it is known not to influence mosquito survival (Liu *et al.* 2010). It should be noted that the PBS solution did not contain any foreign constituents such as dsRNA or dye.

Initially, the most ideal body locality for nano-injection was evaluated simultaneously with mosquito feeding status (starved or sucrose-fed) prior to injection. This was done using 1- to 3-day-old adult females, which were injected with 69 nl PBS either *via* the thorax or abdomen. These conditions were chosen as they have previously been used successfully when conducting nano-injection studies on *An. gambiae* (Blandin *et al.* 2002; Lycett *et al.* 2006; Catteruccia & Levashina 2009; Da Costa *et al.* 2014).

Test mosquitoes were either supplied with a 10 % sucrose solution up until the nano-injection was conducted or were starved by removing the 10 % sucrose solution 12 h prior to nano-injection.

Once the ideal feeding conditions (prior to injection) and body injection locality was determined, using Kaplan-Meier survival analysis, the effect of adult mosquito age and injection volume were assessed. The rate of survival post-injection was assessed over a course of 10 days for all parameters. Survival post-inoculation of all test mosquitoes was compared against control groups of untreated mosquitoes (un-injected), which were fed throughout on a 10 % sucrose solution. The controls were run in parallel with each experiment ($n = 60$).

### *Parameters tested*

i) Effect of feeding condition prior to injection as well as ideal body injection locality on survival post-nano-injection: 1- to 3-day-old mosquitoes, fed on either a 10 % sucrose solution ($n = 60$) or starved 12 h prior to injection ($n = 60$), were injected either *via* the abdomen ($n = 120$) or thorax ($n = 120$) using 69 nl of PBS.

ii) Effect of adult age on survival post-nano-injection: 60 10 % sucrose-fed mosquitos of each age cohort (0, 1, 2 and 3 days of age) were injected with 69 nl of PBS *via* the thorax.

iii) Effect of inoculation volume on survival post-nano-injection: 60 mosquitoes (1-day-old, 10 % sucrose-fed) were injected with 9.2 nl, 18.4 nl, 32.2 nl, and 69.0 nl of PBS, respectively, *via* the thorax.

### *Nano-injection needles*

Injection needles were made from 1.14 × 0.53 mm glass capillaries (Drummond, 3-000-203-G/X) using a needle puller (Leitz, 027-035.002). The needles were used to inject *An. arabiensis* test mosquitoes with PBS. Fine forceps were used to break the point off each of the needles to create a stout, jagged tip that was approximately 20 $\mu$m in diameter. The Nanoinject II (Drummond, 3-000-204) was used for all injections.

*Work station set-up*

The injection equipment was set to the desired output volume, depending on the volume needed (9.2 nl, 18.4 nl, 32.2 nl, and 69.0 nl). The needle was back-filled with mineral oil (Sigma-Aldrich, M5904) allowing for a locked fit. The prepared needle was then placed on the hose of the nano-injector. The needle tip was submerged into the PBS solution allowing for the solution to be drawn into the needle using the fill button.

*Sample preparation*

A styrofoam box was filled with ice. A watch glass was placed on the ice. The adult mosquitoes were cold anaesthetised for 45 s (–20 °C), after which they were placed on the cold watch glass. Each anaesthetised mosquito was moved with forceps to a glass slide placed on the stage of a dissecting microscope, where the injection was administered at room temperature. The fine-tipped forceps were held in one hand, and the nano-injector in the other hand. The forceps were used to support one side of the mosquito, as the mosquito was injected from the other side. The needle tip was carefully inserted into the mosquito either *via* the abdomen (Fig. 1) or thorax (Fig. 2). Once the needle tip was in position, the inject button of the nano-injector apparatus was pressed, which delivered the pre-set volume of PBS solution into the mosquito.

*Post-injection procedures*

A post-injection polystyrene cup was set up at room temperature. A net was placed on top of the cup and secured by an elastic band. A small cotton wool ball, soaked in 10 % sucrose solution, was placed on top of the net. The injected mosquitoes were placed into the post-injection polystyrene

**Fig. 1**. Ventral view of an abdominal inoculation of a female *Anopheles arabiensis* mosquito, using PBS and green dye. Dye was only used for visualisation purposes, and not for experimental data collection.

**Fig. 2**. Lateral view of a thoracic inoculation of a female *Anopheles arabiensis* mosquito, using PBS and green dye. Dye was only used for visualisation purposes, and not for experimental data collection.

cup, where they were left to recover at room temperature. Once the mosquitoes had recovered and were mobile, the cup was placed in the environmentally-controlled insectary with access to a 10 % sucrose solution for the duration of the 10-day observation period. The control group of female mosquitoes were also immobilised for 45 s at –20 °C, after which they were placed in a post-injection cup to recover. This was done in parallel with each experiment.

## RESULTS AND DISCUSSION

### Ideal feeding status and injection locality of adult mosquitoes prior to injection

One day post-injection, sucrose-fed mosquitoes which were intra-thoracically injected showed a significantly higher (Wilcoxon; $P < 0{,}01$) survival rate (87 % survival post-inoculation) when compared to starved intra-thoracically injected mosquitoes (60 % survival post-inoculation), sucrose-fed abdominally injected mosquitoes (57 % survival post-inoculation) and starved abdominally injected mosquitoes (55 % survival post-inoculation) (Fig. 3). Similarly, 10 days post-injection, sucrose-fed intra-thoracically injected mosquitoes showed a significantly higher (Log-rank; $P < 0{,}01$) survival rate (83 % survival post-inoculation) when compared to starved intra-thoracically injected mosquitoes (43 % survival post-inoculation), sucrose-fed abdominally injected mosquitoes (37 % survival post-inoculation) and starved abdominally injected mosquitoes (17 % survival post-inoculation) (Fig. 3).

### Effect of adult age on injection survival

One-day-old sucrose-fed *An. arabiensis* showed the highest survival rate, with 100 % of the mosquitoes initially surviving the injection (Wilcoxon; $P < 0.01$) and 83 % surviving 10 days post-injection (Log-rank; $P < 0.01$) (Fig. 4). Older mosquitoes (2- and 3-day-old) experienced higher mortality after injection, whereas younger mosquitoes (0-day-old, newly emerged) were more vulnerable to the nano-injection. It was observed that 78 % of the newly emerged mosquitoes (6 h old)

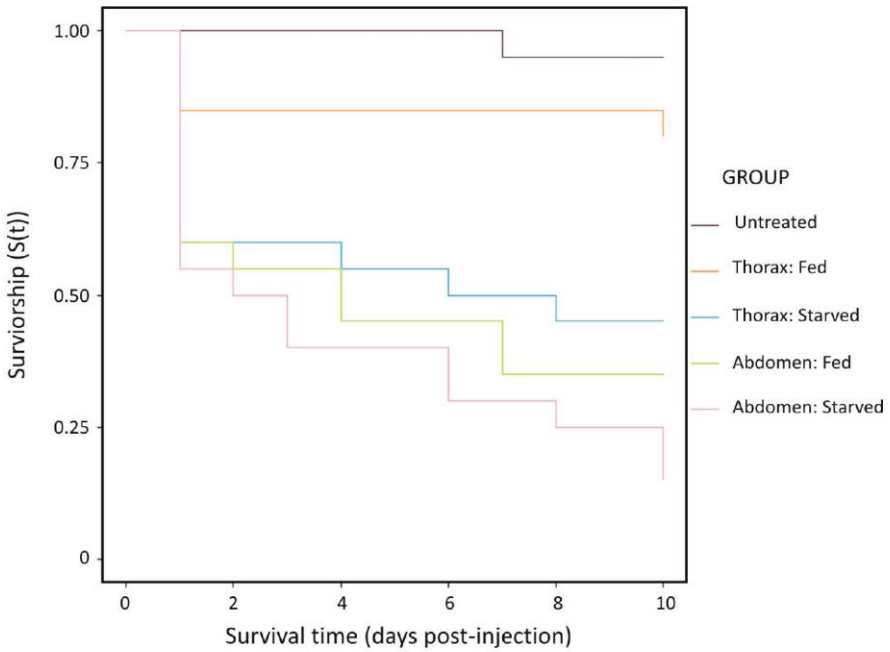


**Fig. 3**. Nano-injection was conducted to empirically determine the optimal injection locality of *Anopheles arabiensis* mosquitoes and the optimal feeding status (starved or sucrose-fed) of adults prior to inoculation. Injection was either administered abdominally or intrathoracically using mosquitoes, which were either starved 12 h prior to inoculation, or allowed to feed on a 10 % sucrose solution. Untreated sucrose-fed mosquitoes were used as a control. The survivability was analysed over a 10-day period, using a Kaplan-Meier survival curve. Survival up to 10 days post-injection was significantly higher using thoracic inoculation into sucrose-fed mosquitoes (d.f. = 4; Log-rank $P < 0.01$, Wilcoxon $P < 0.01$, Tarone-Ware $P < 0.01$).

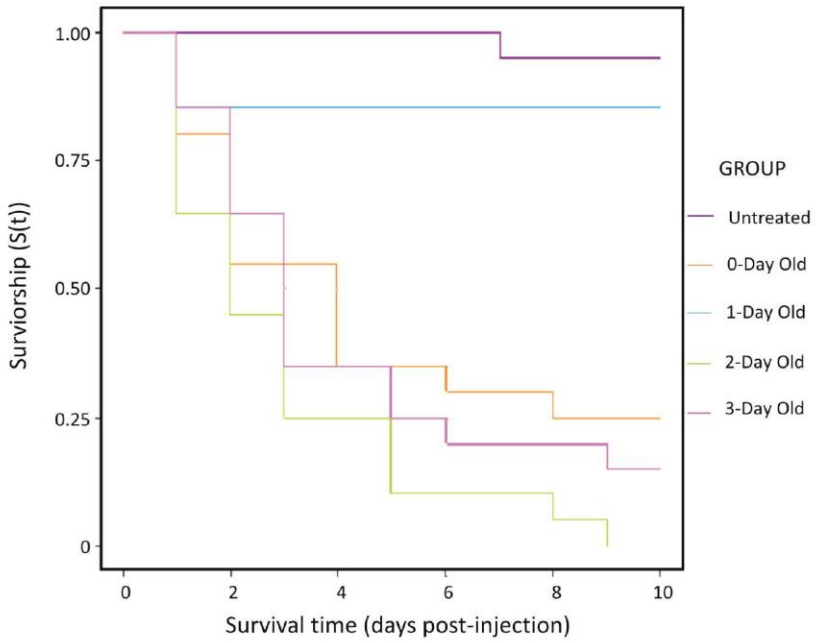


**Fig. 4**. Nano-injection experiments were conducted to determine the effect of *Anopheles arabiensis* adult mosquito age on post-injection survival, over a 10-day period. Four different age groups were tested, 0- (6 h old), 1-, 2- and 3-day-old adult mosquitoes. Untreated sucrose-fed mosquitoes were used as a control. Survival was analysed over a 10-day period, using a Kaplan-Meier survival curve. Survival up to 10 days post-inoculation was significantly higher in the 1-day-old cohort (d.f. = 4; Log-rank $P < 0.01$, Wilcoxon $P < 0.01$, Tarone-Ware $P < 0.01$).

survived one day after the injection, but only 27 % survived for 10 days post-inoculation. When inoculating 3-day-old mosquitoes, 87 % survived one day after the injection, and 17 % survived 10 days post-injection. Two-day-old mosquitoes showed the lowest rate of survival, with 63 % surviving for one day after the inoculation, and only 2 % surviving for 10 days post-inoculation.

**Effect of inoculation volume on survival**

Different inoculation volumes were evaluated on 1-day-old sucrose-fed female *An. arabiensis* mosquitoes, in terms of their effects on post-injection survival (Fig. 5). Mosquitoes injected intra-thoracically with 9.2 nl PBS showed a survival rate of 100 % one day after the injection, decreasing to 90 % surviving 10 days post-injection. Mosquitoes injected intra-thoracically with 18.4 nl PBS showed a 100 % survival rate one day after injection, decreasing to 87 % survival 10 days post-injection. Mosquitoes injected intra-thoracically with 32.2 nl PBS had a 93 % survival rate one day after the injection, decreasing to 83 % survival 10 days post-injection. Inoculation with 69 nl PBS resulted in 80 % of the mosquitoes surviving one day after injection, decreasing to 67 % survival 10 days post-injection. Log-rank, Wilcoxon and Tarone-Ware analyses determined that there was no significant difference in survival in terms of the volume used for nano-injection (d.f. = 4, $P > 0.05$).

This study determined that nano-injection of 1-day-old, sucrose-fed female *An. arabiensis* induced the least mortality if inoculations are injected *via* the thorax. An inoculation volume of 69.0 nl can be used to maximise the volume of inoculate administered to the adult mosquitoes. This combination is similar to the nano-injection technique that is widely used in experimental procedures on *An. gambiae* (Blandin *et al.* 2002; Lycett *et al.* 2006; Catteruccia & Levashina 2009; Da Costa *et al.* 2014). This procedure for inoculation *via* nano-injection in *An. arabiensis* enables a range of follow-on experiments, such as determining the biological function of specific genes to increase biological knowledge on this important and neglected malaria vector. The technique is highly intricate and therefore, both extensive

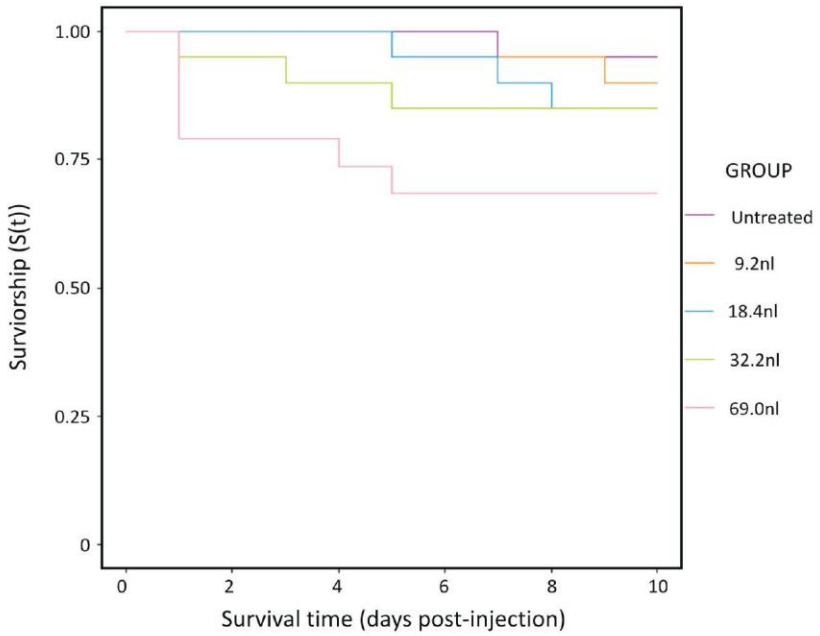


**Fig. 5**. Nano-injection experiments were conducted to empirically determine the optimal inoculation volume delivered to test *Anopheles arabiensis* mosquitoes. Untreated sucrose-fed mosquitoes were used as a control. Survival was analysed over a 10-day period using a Kaplan-Meier survival curve. There was no significant difference in survival between inoculation volumes (d.f. = 4; Log-rank $P = 0.13$, Wilcoxon $P = 0.10$, Tarone-Ware $P = 0.11$).

training and practice are needed to ensure injection success.

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## APPENDIX E

**Letinić, B.D., Dahan-Moss, Y. and Koekemoer, L.L.**, 2020. Characterising the effect of *akirin* knockdown on *Anopheles arabiensis* (Diptera: Culicidae) reproduction and survival, using RNA-mediated interference. *PloS One*, *15*(2), p.e0228576.

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Ms Letinić wrote the first and subsequent drafts of the paper, conducted the experimental work and performed analysis. Contribution of co-authors are detailed in the manuscript.

PLOS ONE

RESEARCH ARTICLE

# Characterising the effect of *Akirin* knockdown on *Anopheles arabiensis* (Diptera: Culicidae) reproduction and survival, using RNA-mediated interference

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## Abstract

*Anopheles arabiensis* is an opportunistic malaria vector that rests and feeds outdoors, circumventing current vector control methods. Furthermore, this vector will readily feed on animal as well as human hosts. Targeting the vector, while feeding on animals, can provide an additional intervention for the current vector control activities. Agricultural animals are regularly vaccinated with recombinant proteins for the control of multiple endo- and ecto-parasitic infestations. The use of a Subolesin-vaccine showed a mark reduction in tick reproductive fitness. The orthologous gene of *Subolesin*, called *Akirin* in insects, might provide a valuable species-specific intervention against outdoor biting *An. arabiensis*. However, the biological function of this nuclear protein has not yet been investigated in this mosquito. The effects on *An. arabiensis* lifetable parameters were evaluated after *Akirin* was knocked down using commercial small-interfering RNA (siRNA) and *in vitro* transcribed double-stranded RNA (dsRNA). The siRNA mediated interference of *Akirin* significantly reduced fecundity by 17%, fertility by 23% and longevity by 32% when compared to the controls in the female mosquitoes tested. Similarly, dsRNA treatment had a 25% decrease in fecundity, 29% decrease in fertility, and 48% decrease in longevity, when compared to the control treatments. Mosquitoes treated with Akirin dsRNA had a mean survival time of 15-days post-inoculation, which would impact on their ability to transmit malaria parasites. These results strongly suggest that Akirin has a pleiotropic function in *An. arabiensis* longevity and reproductive fitness.

## Introduction

Vector-borne diseases cause more than 700,000 deaths annually [1]. The mosquito-borne disease, malaria, is accountable for 60% of these annual deaths [2]. In Africa, malaria is caused by the bite of a *Plasmodium*-infected female mosquito mainly belonging to the *Anopheles funestus* group or the *Anopheles gambiae* complex. The *Anopheles gambiae* complex is comprised of eight species, two of which are minor malaria vectors (*An. merus* and *An. melas*), while three

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are major malaria vectors (*An. gambiae s.s.*, *An. coluzzii*, and *An. arabiensis*) [3, 4]. The main malaria vector in South Africa is *An. arabiensis* [5].

In South Africa, malaria primarily occurs in the Limpopo, Mpumalanga and KwaZulu-Natal provinces. While these provinces implement well-coordinated malaria control operations, vector control is based predominantly on the application of indoor residual spraying [6]. *Anopheles arabiensis* mosquitoes are seen as an opportunistic species, as they show variations in resting behaviour (endophilic and exophilic) and feeding behaviour (endophagic, exophagic, anthropophilic and zoophilic) [3] when compared to other *Anopheles* malaria vector species. This makes it practically impossible to eliminate *An. arabiensis* using current control methods, contributing to the ongoing transmission of residual malaria in South Africa [5].

This problem is compounded by the fact that insecticide resistance is increasing in the vector population, which is most likely due to increased selection pressure [7]. In South Africa, the *An. arabiensis* population has shown resistance to DDT (Dichlorodiphenyltrichloroethane), pyrethroids, and carbamates, especially in northern KwaZulu-Natal [7–9]. This places tremendous pressure on the goal of eliminating malaria from South Africa by 2030 [2], and necessitates the need for the development of additional vector control interventions. However, the identification of additional novel interventions is largely dependent on the identification of new target sites or biological pathways [10].

Akirin is a highly conserved nuclear transcription co-factor [11], that plays a crucial role in innate immunity [12]. The innate immune system is comprised of two distinct signalling pathways, namely the immune deficiency (Imd) pathway and the Toll pathway. Akirin regulates NF-kB dependent transcription in the Imd pathway, as it is required at the level of the transcription factor Relish [12]. RNAi-mediated gene knockdown of *Akirin* in *Drosophila melanogaster* has been shown to impair Imd signal transduction and enhance sensitivity to Gram-negative bacterial infection [12]. *Akirin* knockdown in *An. coluzzii* (historically called *An. gambiae* M-molecular form [4]) showed an increased susceptibility to *P. berghei* infection, supporting its role in mosquito immunity [13, 14].

Akirin also plays a critical role in processes unrelated to immunity, such as embryonic development [12]. Knockdown of its ortholog in ticks, *Subolesin*, resulted in several phenotypic deleterious effects [15, 16]. This included significantly diminished feeding capabilities of engorged ticks, resulting in a notable decline in tick mass [17]. In addition to these phenotypic changes, developmental abnormalities such as tissue damage, failure of nymph metamorphosis, diminished vectorial capacity, reduced survival, and reduction reproductive fitness were also observed [17, 18].

The highly conserved nature of Akirin provides a valuable species-specific intervention against *An. arabiensis*. However, the biological function of this nuclear protein has not yet been investigated in this mosquito. The effects on *An. arabiensis* lifetable parameters were evaluated, with specific focus on vector fecundity, fertility, and longevity.

## Materials and methods

### Biological material

A laboratory strain of *An. arabiensis* mosquitoes (MBN) was used. This strain was colonised in 2002, from wild material collected in Mamfene, northern KwaZulu-Natal, South Africa [8]. It is maintained in the Botha De Meillon Insectary, at the Vector Control Reference Laboratory (VCRL) of the National Institute for Communicable Diseases (NICD) in Johannesburg, under standard insectary conditions of 80% humidity, 25˚C, and a 12-hour day/night cycle with 45-minute dusk/dawn transitions [19]. All adult mosquitoes were sustained on a 10% sucrose solution diet. Blood meals were provided to mated female mosquitoes via routine guinea pig

blood feeding. Blood feeding protocol and ethics were reviewed and approved by the National Health Laboratory Service (NHLS), Animal Ethics Committee (AEC) (AESC: 1993–047).

Guinea pig colony are maintained at the NHLS animal until issued. Original breeding stock was bought from Harlan UK Labs. Guinea pigs are kept in standard laboratory cages at a temperature range of 21˚C (+/- 2˚C), with a light/dark cycle of 12 hours. Cages are cleaned three times per week. Guinea pigs are provided with a bed of pine shavings, with added hay/eragrostis, and are provided with commercial rabbit pellets and water ad-lib. Water is supplemented with vitamin C (three times a week). To minimize stress on the animals, staff and routine activities are kept constant in a strict "low noise" environment, preventing unnecessary stress. Animal injections and veterinary procedures were strictly conducted by trained South African Veterinary Council (SAVC) registered staff. Prior to blood feeding, guinea pigs are anaesthetised to prevent distress of the animals.

## RNA interference

RNA interference is a technique that uses exogenous double-stranded RNA (dsRNA) to repress the expression of endogenous messenger RNA (mRNA), subsequently causing loss of gene transcript [20]. Two separate techniques were used to repress the expression of endogenous *Akirin* mRNA in *An. arabiensis* mosquitoes. The first technique used commercially available small interfering RNAs (siRNAs), that were synthesised by ThermoFisher using Ambion® *in vivo* chemical modifications to ensure minimal off-target activity. Putative siRNAs were selected based on the *Silencer*® Select algorithm, that was used to analyse the full coding sequence of each gene (See S1 Table for siRNA sequences).

RNAi-mediated knockdown, using Ambion® siRNA, was optimised using Glyceraldehyde 3-phosphate dehydrogenase (GapDH)-specific siRNA as a positive control (ThermoFisher, s541529). *GapDH* was significantly down-regulated by a mean factor of 0.16 (S.E. range 0.11–0.28) ($p = 0.03$) when conducting qPCR. Ambion® *in vivo* custom-designed siRNA was used to target Akirin (ThermoFisher, s541552), while the Ambion® pre-designed Mouse-ß2m siRNA (*in vivo)* was used as a negative control (ThermoFisher, s62844).

The second technique used *in vitro* transcribed dsRNA that was synthesised using RNA that was isolated from 1-day-old untreated female *An. arabiensis* mosquitoes. RNA was extracted using Trizol™ (Invitrogen, 15596026) and quantified using a NanoDrop™ spectrophotometer. The integrity of the RNA was assessed using a 1% non-denaturing agarose gel, TBE (Tris/Borate/EDTA) [21]. The gel electrophoresis was conducted at 70V for 30 minutes, to ensure minimal RNA degradation. The isolated RNA was reverse-transcribed into complementary DNA (cDNA) using the QuantiTect® reverse-transcription kit (Qiagen, 205310).

The cDNA was amplified using primers designed to synthesise Akirin dsDNA [13]. The forward primer ($^{5'}$GGTACTTTGGCAGTCGTTGTAGTTGC$^{3'}$) and the reverse primer ($^{5'}$GGTACTCACCTGCTTGAAGGTGAACA$^{3'}$) contained T7 promoters ($^{5'}$TAATACGACTCACTATAG $^{3'}$) that were appended at the 5' prime end of the sequence, to allow for *in vitro* transcription to take place. Polymerase chain reaction (PCR) was performed using cDNA (250ng), 1x PCR buffer (10mM Tris-HCl, 5mM KCl), 1mM dNTP's, 3mM $MgCl_2$, 0.4μM forward primer, 0.4μM reverse primer, and 0.1U *Taq* polymerase, which was made up to a final volume of 25μl using nuclease-free water. PCR was conducted by heating the sample to 94˚C for 5 minutes, followed by 40 PCR cycles (94˚C for 30 seconds, 52˚C for 1 minute, 72˚C for 1 minute), and a final extension at 72˚C for 5 minutes. PCR products were purified using the QIAquick® PCR purification kit (Qiagen, 28104), as per the manufacturer's specifications. The purified PCR products were quantified using the NanoDrop™ spectrophotometer, after

which the integrity and size of the purified PCR products (250ng) were examined on a 1% agarose gel (75 minutes, 110V), TAE (Tris/Acetate/EDTA), prior to *in vitro* transcription.

The MEGAscript® RNAi kit (Invitrogen, AM1626) was used to assemble the transcription reaction, by generating two complementary RNA transcripts. In a 20μl reaction, 1-fold of ATP, CTP GTP, UTP, T7 enzyme mix and reaction buffer was added to 1μg dsDNA template. The sample reactions were incubated at 37˚C for 4 hours. Nuclease digestion was performed to digest residual template DNA and ssRNA. Nuclease digestion was conducted by adding 2.5x digestion buffer, 2μl of DNaseI, 2μl of RNase, and 21μl nuclease-free water to the *in vitro* transcribed dsRNA product. This reaction was incubated for 1 hour at 37˚C to allow for the template DNA to digest. The digested DNA, free nucleic acids, and residual proteins were removed from the dsRNA using the filter cartridge provided in the MEGAscript® RNAi kit, as specified by the manufacturer. The eluted purified *in vitro* transcribed dsRNA was quantified using the NanoDrop™ spectrophotometer, and the integrity was assessed on a 1% agarose gel, TAE (75 minutes, 110V).

## Inoculation

The Nanoinject II (Drummond, 3–000204) was used to conduct RNAi inoculation on 1-day-old female *An. arabiensis* mosquitoes [22]. Cold anesthetised mosquitoes were inoculated with 69nl (3mg/ml) of Akirin dsRNA, Akirin siRNA or Mouse-ß2m siRNA. A subset of cold anesthetised mosquitoes injected with PBS was used as a handling control, while a subset of mosquitoes that were not subjected to inoculation, but had undergone cold anesthetisation, were used as an untreated control. Fatalities as a result of the injection procedure were minimal. However, if a fatality occurred immediately after injection, the mosquito was discarded from the analysis.

Inoculations were conducted using a total of 300 female mosquitoes per each treatment. Fifty mosquitoes were randomly selected from each treatment for quantitative-PCR (qPCR) analysis (10 mosquitoes per replicate, 5 replicates), while 150 female mosquitoes were randomly selected for longevity analysis (30 mosquitoes per replicate, 5 replicates). The remaining 100 female mosquitoes from each treatment were used to analyse vector fertility and fecundity (20 mosquitoes per replicate, 5 replicates). All treatments were carried out in 32.5cm$^3$ insect rearing cages (BugDorm®, 211476).

## Quantitative-PCR

Previous *Akirin* knockdown studies showed a 16–40% decrease in Akirin expression in the midgut of the mosquito, while the remaining tissues showed a 25–65% decrease in Akirin expression [13]. For this reason, RNA was extracted from whole mosquito samples three days post-RNAi inoculation. RNA was reverse-transcribed into cDNA using the QuantiTect® reverse-transcription kit. Quantitative-PCR was conducted using 1x iQ™ SYBR® Green Supermix (Bio-Rad, 170–8887), 0.48μM forward primer, 0.48μM reverse primer, and 100ng cDNA template, which was made up to a final volume of 25μl using nuclease-free water. All samples were amplified using the C1000™ thermal-cycler, Bio-Rad CFX96 Touch™ real-time PCR detection system. PCR amplification was performed by preheating the reaction to 94˚C for 2 minutes, followed by 40 PCR cycles (94˚C for 30 seconds, 62˚C for 30 seconds, 72˚C for 40 seconds), and a final PCR extension of 10 minutes at 72˚C.

The relative expression ratios of the *Akirin* knockdown samples were measured in comparison to the *Mouse-ß2m* knockdown samples using REST© analysis (relative expression software tool). The relative expression ratios of *Mouse-ß2m* knockdown samples were also compared to the PBS and untreated samples in each treatment, to ensure that the results being observed

were gene-specific. Gene expression was normalised using *RPS7* and *RPS26* as reference genes (See S2 Table for primer sequences).

### Fecundity and fertility

Mosquito fecundity and fertility were analysed post-inoculation using a total of 100 female mosquitoes per treatment (20 mosquitoes per replicate, 5 replicates). The female mosquitoes were placed in cages with untreated 2-day-old male mosquitoes (20 mosquitoes per cage) for a period of 4-days to allow mating to take place, after which the male mosquitoes were removed from the cages. Two subsequent blood meals were offered to the mated females over five days [23]. After receiving the second blood meal, each female was transferred into a separate paper cup (8cm diameter, 9cm height), with a net fastened over the top of the cup using an elastic band. Approximately 20ml of distilled water was poured into each cup. Mosquitoes were maintained on a 10% sucrose solution until oviposition had taken place. Vector fecundity was determined by calculating the mean number of eggs laid per female. The hatch rate was monitored over 10-days. Eggs which did not hatch 10-days post oviposition were deemed infertile. Vector fertility was determined by calculating the mean percentage of hatchlings per female.

### Longevity

Longevity was assessed to determine whether *Akirin* knockdown affected *An. arabiensis* survival. Longevity was evaluated using a total of 150 female mosquitoes per treatment (30 mosquitoes per replicate, 5 replicates). The rate of survival was monitored until 100% mortality was reached for all five treatments (Akirin dsRNA, Akirin siRNA, Mouse-ß2m siRNA, PBS, and untreated). A Kaplan-Meier survival curve was constructed using Statistix 10, where the probability of survival for each treatment was displayed as horizontal lines on the curve. The vertical line's distance between the horizontal lines illustrated the change in cumulative probability, which changed when a death occurred [24].

### Statistical analysis

All statistical analysis was performed at a 95% confidence interval. A one-way analysis of variance (ANOVA) and a Tukey HSD post-hoc test were used to compare the mean fecundity and fertility between the treatments. The Log-rank test was used to determine whether there was a statistically significant difference in vector longevity between the various treatments.

## Results

### Quantitative-PCR

Samples treated with Akirin-specific dsRNA showed a significant reduction in *Akirin* expression, by a mean factor of 0.76 (S.E. range is 0.65–0.88) ($p = 0.02$), when compared to the negative control samples (Fig 1). As expected, no significant change was detected amongst the control treatments, confirming that the change in relative expression was gene-specific. The change in *Akirin* expression was also assessed in the samples treated with Akirin-specific siRNA. Although the Akirin-specific siRNA treated samples showed a reduction in *Akirin* expression, by a mean factor of 0.82 (S.E. range is 0.65–0.99) ($p = 0.06$) when compared to the negative control samples, the change in expression was not statistically significant at a 95% confidence interval.

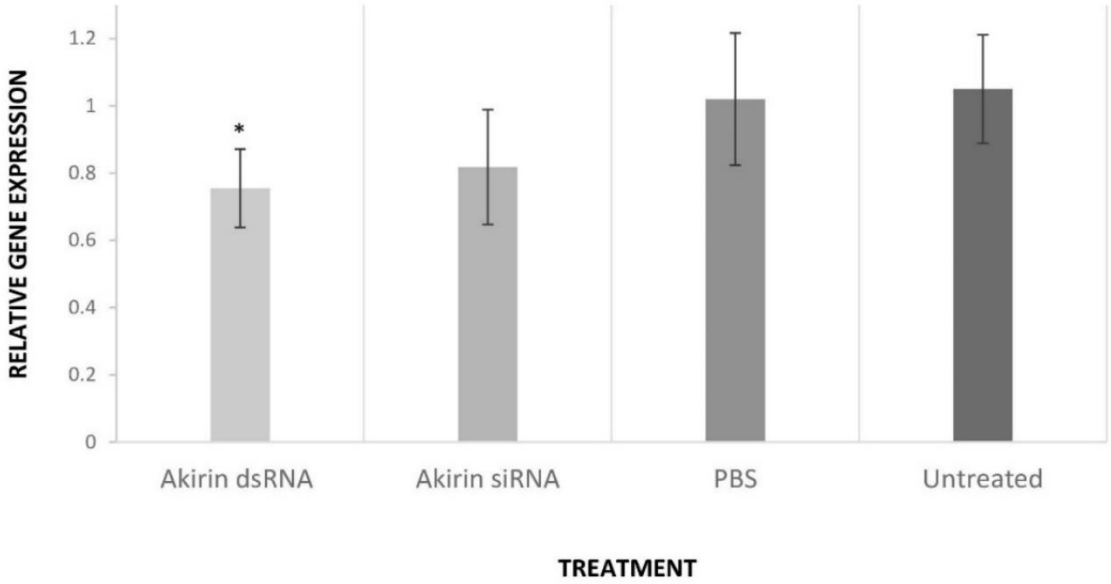


**Fig 1. The change in *Akirin* relative expression assessed 3-days post-RNAi treatment.** The relative expression of *Akirin* was significantly downregulated by a mean factor of 0.76 ($p = 0.02$), when comparing the expression ratios between the Akirin dsRNA treated samples and the negative control samples. The relative expression of *Akirin* remained unchanged when comparing the expression ratios of the negative control samples (Mouse-ß2m siRNA) to the PBS treated samples ($p = 0.78$) and untreated samples ($p = 0.83$).

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## Fecundity and fertility

Mosquito fecundity and fertility were assessed between the various treatments, to determine whether *Akirin* knockdown had an impact on *An. arabiensis* oviposition (Fig 2). Mosquitoes treated with Akirin-specific siRNA and Akirin-specific dsRNA had a 1.3-fold (17%) and 1.4-fold (25%) decrease in fecundity respectively, when compared to the control treatments (one-way ANOVA: $p<0.01$, F = 16.5, DF = 4). The control treatments had a mean fecundity of approximately 110 eggs laid per female mosquito, while those treated with Akirin-specific siRNA and Akirin-specific dsRNA had mean fecundity of 91 and 82 eggs laid per female respectively. Mosquitoes treated with Akirin-specific siRNA and Akirin-specific dsRNA had a further 1.2-fold (19%) and 1.4-fold (26%) decrease in fertility respectively, when compared to the control treatments (one-way ANOVA: $p<0.01$, F = 63.4, DF = 4). The control treatments had a mean hatch rate of 92%, while those treated with Akirin-specific siRNA and Akirin-specific dsRNA had a mean hatch rate of 72% and 65% respectively.

## Longevity

The results of the longevity experiments are shown in Fig 3. The control treatments had a mean survival time of 28-days, while the mosquitoes treated with Akirin siRNA and Akirin dsRNA had a mean survival time of 20-days and 15-days post-inoculation respectively. At 15-days post-inoculation, mosquitoes treated with Akirin siRNA had a mortality of 35%, while the mosquitoes treated with Akirin dsRNA had a mortality of 52%. This was significantly higher than the controls (Log-rank: $\chi^2 = 78$, $p<0.01$, DF = 4), as the mosquitoes treated with Mouse-ß2m siRNA and PBS had a mortality of 20%, and the untreated mosquitoes had a mortality of 12%. Mosquitoes treated with Akirin siRNA and Akirin dsRNA reached 100% mortality 33-days and 25-days post-inoculation respectively, while the control treatments reached

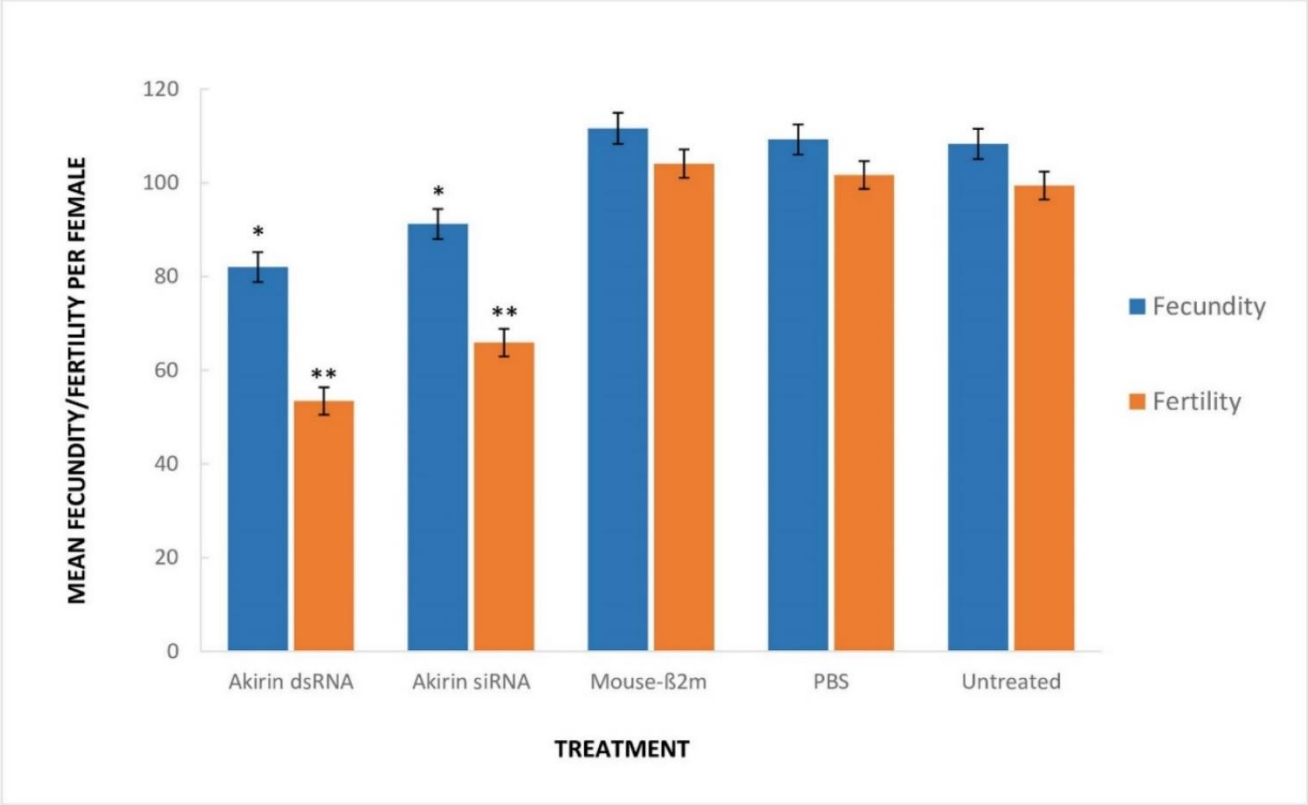


**Fig 2. Mean vector fecundity (blue) and fertility (orange) per treatment.** *Akirin* knockdown mosquitoes had a reduced mean fecundity by 17–25% (one-way ANOVA: $p<0.01$, F = 16.5, DF = 4), and a reduced mean fertility by 23–29% (one-way ANOVA: $p<0.01$, F = 63.4, DF = 4), when compared to the control treatments. This was a 19–26% decrease in hatch rate percentage. No significant change in fecundity/fertility was observed between the control treatments. The asterisks indicate a significant change from the control treatments.

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100% mortality after 47-days. When compared to the control treatments, the mosquitoes treated with Akirin siRNA had a 32% decrease in longevity overall, while the mosquitoes treated with Akirin dsRNA had a 48% decrease in longevity in total (Log-rank: $\chi^2 = 154$, $p<0.01$, DF = 4).

## Discussion

Characterising the effect of *Akirin* knockdown on *An. arabiensis* provided insight into its pleiotropic function in insects, and its value as a species-specific intervention against *An. arabiensis*. Two different knockdown molecules (dsRNA and siRNA) were used to repress the expression of endogenous *Akirin* mRNA in *An. arabiensis* mosquitoes. Regardless of the molecule used for repression, a statistically significant change in vector fecundity, fertility, and longevity was observed. This was consistent with the results obtained when *Akirin* was downregulated in *An. coluzzii*, where fecundity was reduced by 52% and survival by 14% [13]. Although Akirin is known to be involved in various other physiological and developmental pathways, no other visible phenotypic differences were observed between the *Akirin* knockdown mosquitoes and the controls.

The *in vitro* transcribed exogenous dsRNA was, however, more efficient in repressing *Akirin* expression in *An. arabiensis* than the commercially available exogenous siRNA. This

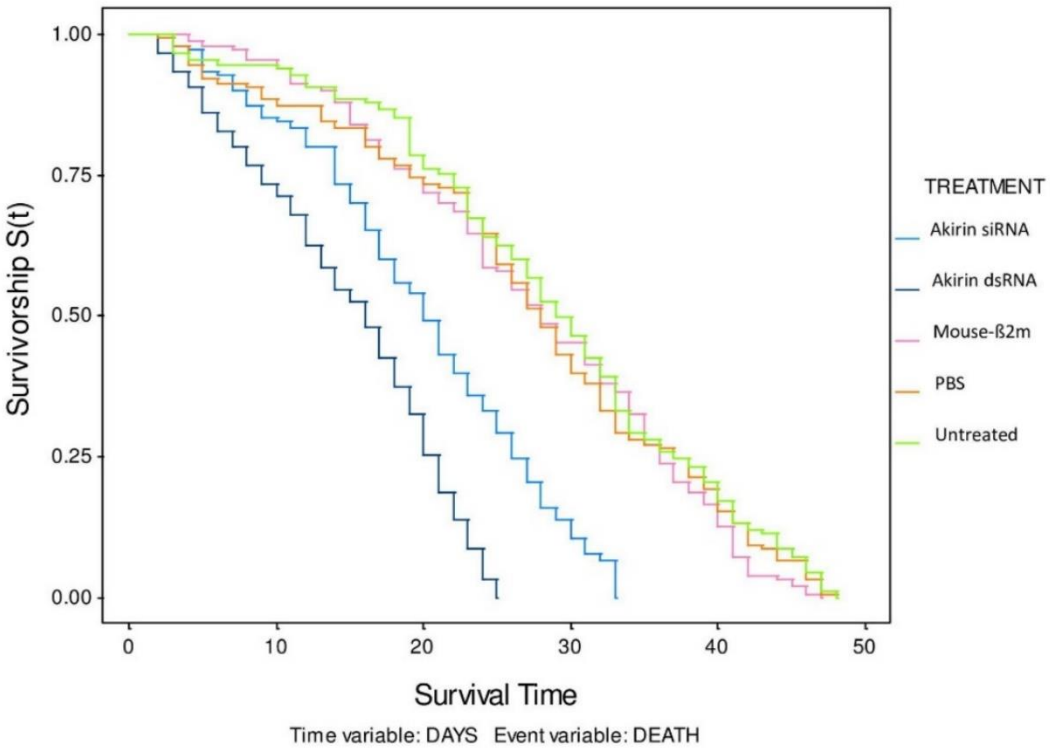


**Fig 3. Kaplan-Meier survival analysis of Akirin knockdown (blue), Mouse-ß2m (pink), PBS (orange) and untreated (green) mosquitoes.** Female mosquitoes treated with Akirin siRNA and Akirin dsRNA reached 100% mortality 15 and 23-days (respectively) before the control treatments, which conveyed a 32–48% decrease in vector longevity (Log-rank: $\chi^2 = 154$, $p<0.01$, DF = 4).

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was due to improved absorption uptake of the dsRNA [25], as well as asymmetry in the assembly of the RNAi enzyme complex [26]. Although less efficient, deleterious effects were still apparent when assessing the lifetable parameters in the mosquitoes treated with Akirin-specific siRNA. This suggested that *Akirin* expression could have recovered shortly after being repressed however, its downregulation still had downstream physiological effects in *An. arabiensis* [27].

Akirin is required in the Imd pathway [14]. Upon immune challenge, Akirin binds to BAP60, which is a component of the Brahma (SWI/SNF) ATP-dependent chromatin-remodelling complex [12]. The Akirin-BAP60 complex then binds to Relish, which is NF-kB transcription factor, forming a link between Relish and the BAP complex on the promoter of a subset of NF-kB target genes [12, 15, 28]. This link allows for efficient anti-microbial peptide synthesis [29]. The downregulation of Akirin impairs the gene expression of several antimicrobial peptide-coding genes, weakening the innate immune defence required for survival [29].

Mosquitoes treated with Akirin-specific dsRNA had a 54% reduction in mean survival time in relation to the controls. The control treatments had a mean survival time of 28-days, while the mosquitoes treated with Akirin-specific dsRNA had a mean survival time 15-days post-inoculation. This is a significant finding since *Anopheles* mosquitoes are able to transmit malaria parasites 14-days post gametocyte infection [30]. This provides the premise for future vaccine development using recombinant *An. arabiensis* Akirin as a potential species-specific antigen for the control of *An. arabiensis*.

The recombinant Akirin vaccine would be administered to various reservoir hosts, to elicit an antibody response against the nonself epitopes [13, 31]. Mosquitoes feeding on the vaccinated hosts would ingest antibodies specific to the target antigen. Once ingested, the antibodies would be transported across the gut barrier into the haemolymph, entering the mosquito's cells [18, 32]. The antibodies would interact with cytosolic Akirin preventing translocation to the nucleus. This would inhibit Akirin from exerting its regulatory function, causing deleterious effects within the vector [13, 18, 31, 32].

Recombinant Akirin antigens have previously been tested against several Culicidae species, with varying results. The ingestion of anti-Akirin antibodies, from mice vaccinated with recombinant *Aedes albopictus* Akirin, caused a 29% decrease in longevity of the European malaria vector, *An. atroparvus* [33]. Similarly, vector longevity was also reduced in *Ae. caspius* (29%), *Ae. albopictus* (17%) and *Culex pipiens* (11%) [33, 34]. However, vector longevity was not affected when *An. coluzzii* and *Ae. aegypti* ingested recombinant *Ae. albopictus* anti-Akirin antibodies [13, 33]. These differences were attributed to the physiological difference between the various species [34], which may be overcome by using a species-specific antigen approach.

The data presented here, as well as the opportunistic feeding behaviour characteristic of *An. arabiensis* females, provides the impetus to evaluate the use of recombinant Akirin vaccines in this major African malaria vector.

## Conclusions

*Akirin* knockdown in *An. arabiensis* female mosquitoes significantly reduced longevity, fecundity and fertility, suggesting that Akirin has a pleiotropic function in *An. arabiensis* survivorship and reproductive fitness.

## Supporting information

**S1 Table. siRNA sequences used to conduct RNAi.**
(DOCX)

**S2 Table. Quantitative-PCR primer sequence.**
(DOCX)

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## APPENDIX F

**Letinić, B.D., Contreras, M., Dahan-Moss, Y., Linnekugel, I., de la Fuente, J. and Koekemoer, L.L.**, 2020. Characterising the effect of three different recombinant Akirin vaccines on *Anopheles arabiensis*. *Parasites and Vectors* (submitted and under review).

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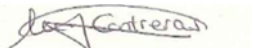
| 2nd Author | Marinela Contreras |  | 27 July 2020 |
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**Comments by Primary Supervisor:**

Ms Letinić wrote the first and subsequent drafts of the paper, conducted the experimental work and performed analysis. Contribution of co-authors are detailed in the manuscript.

# Characterising the effect of three different recombinant Akirin vaccines on *Anopheles arabiensis*

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**Abstract**

**Background:** *Anopheles arabiensis* is an opportunistic malaria vector that rests and feeds outdoors, circumventing current indoor vector control methods. Furthermore, this vector will readily feed on animal as well as human hosts. Targeting this vector while feeding on animals, can provide an additional intervention for the current vector control activities. Previous results have shown efficacy of Subolesin/Akirin ortholog

vaccines for the control of ectoparasite infestations. This made Akirin a potential antigen for vaccine development against *An. arabiensis*.

**Methods:** The efficacy of three antigens, namely recombinant Akirin from *An. arabiensis,* recombinant Akirin from *Aedes albopictus* and recombinant Q38 (Akirin/Subolesin chimera), were evaluated as novel interventions for *An. arabiensis* vector control. Immunisation trials were conducted based on the concept that mosquitoes feeding on vaccinated balb/c mice would ingest antibodies specific to the target antigen to affect its function within the vector.

**Results:** All three antigens successfully reduced *An. arabiensis* survival and reproductive capacities, with a vaccine efficacy of 68-73%.

**Conclusions:** These results were the first to show that hosts vaccinated with recombinant Akirin vaccines could develop a protective response against the outdoor malaria transmission vector, thus providing a step towards to the development of a novel target for *An. arabiensis* vector control.

**Keywords:** malaria; vector control; Akirin; Subolesin; immunisation; recombinant proteins

## 1. Background

Traditional control methods for arthropod vectors are mainly based on the use of chemical insecticides [1]. Insecticide resistance due to increased selection pressure has necessitated the need to develop new classes of insecticides or develop other novel control interventions. Novel approaches which exploit the natural behaviour of vectors can be used to target these ectoparasites. The opportunistic African malaria vector, *Anopheles arabiensis,* will readily feed on animal as well as human hosts [2]. Targeting the vector while feeding on animals, can provide an additional intervention for the current vector control strategies.

Agricultural animals are regularly vaccinated with recombinant vaccines for the control of multiple endo- and ecto-parasitic infestations [3-5]. These recombinant vaccines contain protective

55 antigens that develop antigen-specific antibodies in immunised hosts [6]. Arthropod vectors ingest
56 these antibodies when feeding on such hosts. The antibodies interact and disrupt the function of the
57 target antigen in the arthropod vector, resulting in physiological changes that affect vector biology
58 [7,8].
59
60 This concept was first demonstrated when blood-feeding ectoparasites (*Dermacentor andersoni*)
61 were adversely affected when feeding on hosts that had been vaccinated with crude extracts of these
62 arthropods [9]. Subsequently, the first arthropod vaccines, namely Gavac™ and TickGARD$^{PLUS}$, were
63 manufactured and distributed after the *Rhipicephalus microplus* gut antigen (BM86) was used to
64 control tick infestations, through cattle immunisation [3,6,10-13]. It has since been proposed that
65 vaccination, using evolutionary conserved protective antigens, could be used to develop multi-target
66 vaccines directed at the control of several vector species [14]. However, the limiting step in the
67 development of such vaccines is the identification of these antigens [15]. Using expression library
68 immunisation, an evolutionary conserved protective antigen, Subolesin, was discovered in *Ixodes*
69 *scapularis* [16-18].
70
71 Immunisation, using recombinant Subolesin, caused several deleterious effects in engorged ticks
72 including diminished vectorial capacity, reduced oviposition, and reduced vector survivorship
73 [17,19]. Feeding capabilities were also adversely impacted, resulting in a decline of tick mass [18].
74 Additionally, developmental abnormalities were observed, such as the failure of nymph
75 metamorphosis, as well as tissue damage in various tick species [17,20]. The insect ortholog of
76 Subolesin, known as Akirin, was discovered after phylogenetic analysis showed a high degree of
77 sequence similarity between these two proteins [21-23]. Akirin is a conserved nuclear transcription
78 co-factor involved in multiple biological processes, such as its role in innate immunity [21].
79

80 The innate immune system is comprised of two distinct signalling pathways, namely the
81 immune deficiency (IMD) pathway and the Toll pathway [24]. The role of Akirin was demonstrated
82 when RNA interference (RNAi) mediated gene knockdown impaired IMD pathway signalling and
83 enhanced sensitivity to Gram-negative bacterial infection in *Drosophila melanogaster* [21]. Akirin
84 regulates NF-kB dependent gene expression in the IMD pathway, as it is required at the level of the
85 transcription factor Relish [21,25,26].
86
87 Generation of homozygous null Akirin *D. melanogaster* mutants resulted in an embryonic lethal
88 phenotype, indicating that Akirin also plays a critical role in processes unrelated to immunity, such
89 as embryogenesis [21]. RNAi-mediated knockdown of *Akirin* reduced survival and reproductive
90 capacities in several Culicidae species [14], making Akirin a potential antigen for vaccine
91 development against mosquito populations.
92
93 Preliminary immunisation studies were evaluated on *Aedes caspius*, *An. atroparvus,* and *Culex*
94 *pipiens* mosquitoes [14]. Immune serum was acquired from New Zealand white rabbits that were
95 vaccinated with recombinant Akirin from *Ae. albopictus* (Akirin$^{albopictus}$). The hyperimmune serum was
96 administered to the various mosquito species via a solution embedded cotton wool, to determine the
97 effect of antigen-specific antibodies on mosquito life parameters. The ingestion of the antigen-specific
98 antibodies decreased mosquito survivorship [14], which provided the impetus needed for
99 subsequent recombinant Akirin$^{albopictus}$ immunisation studies.
100
101 Mice were subsequently vaccinated with recombinant Akirin$^{albopictus}$ to evaluate the effect of
102 antigen-specific antibodies on *Ae. albopictus, Ae. aegypti* and *An. coluzzii* life-parameters, though the
103 results from these investigations varied [27,28]. The ingestion of Akirin$^{albopictus}$ antibodies diminished
104 *Ae. albopictus* and *An. coluzzii* reproductive capacities however, it failed to reduced vector
105 survivorship [27,28]. On the contrary, the ingestion of Akirin$^{albopictus}$ antibodies failed to reduce both

106 vector survivorship and reproductive capacities in *Ae. aegypti* [27]. The difference in vaccine efficacy
107 was attributed to physiological difference between the various species [27]. Another antigen was
108 subsequently evaluated for the control of *Ae. albopictus* mosquito populations. The Q38 antigen was
109 a chimera that was designed by combining protective epitopes from *I. scapularis* Subolesin and *Ae.*
110 *albopictus* Akirin [29]. Ingestion of Q38 antigen-specific antibodies reduced *Ae. albopictus* reproductive
111 capacities as well as survivorship, demonstrating the possibility of using Q38 as a multi-target
112 arthropod vaccine [29].
113
114 The effects of Akirin immunisation have yet to be evaluated in *An. arabiensis,* which is the main
115 malaria vector in South Africa [30] and other sub-Saharan countries [1]. RNA-interference mediated
116 knockdown of *akirin* in *An. arabiensis,* reduced vector survivorship and reproductive capacities [31],
117 supporting the use of Akirin as a potential antigen for vaccine development against this malaria
118 vector. To determine whether Akirin could represent a novel target for the control of *An. arabiensis*,
119 the efficacy of recombinant Akirin$^{albopictus}$ [27], and recombinant Q38 [29] were evaluated on *An.*
120 *arabiensis* life-parameters. However, *Aedes* are vectors for diseases unrelated to malaria, namely
121 dengue fever, yellow fever, chikungunya, and Zika virus among many others. An additional genera-
122 specific recombinant antigen was therefore evaluated on *An. arabiensis,* namely recombinant Akirin
123 from *An. arabiensis* (Akirin$^{arabiensis}$). Vector survival, fertility, or fecundity were significantly affected
124 highlighting that, vaccination using these protective antigens could be developed further as a
125 supplementary tool for *An. arabiensis* vector control.
126

127 **2. Methods**

128

129 *2.1 Biological Material*

130

A laboratory strain of *An. arabiensis* mosquitoes (MBN) was used. This strain was colonised in 2002, from wild material collected in Mamfene, northern KwaZulu-Natal, South Africa [32]. It is maintained in the Botha De Meillon Insectary, at the Vector Control Reference Laboratory (VCRL) of the National Institute for Communicable Diseases (NICD) in Johannesburg, under conditions of 80% humidity, 25 °C, and a 12-hour day/night cycle with 45-min dusk/dawn transitions [33]. All adult mosquitoes were sustained on a 10% sucrose solution diet. Two subsequent blood meals were provided to mated female mosquitoes, 5-days apart, from immunised balb/c mice.

The balb/c colony is sourced and maintained at the South African Vaccine Producers (SAVP) animal unit of the NICD, at a temperature range of 21 °C (±2 °C), with a 12-h day/night cycle. The mice were housed in standard laboratory cages, on a bed of pine shavings and added shredded paper for enrichment, bedding was changed twice a week. The mice were sustained on a diet consisting of commercial mouse pellets as well as water *ad-lib*. An animal monitoring sheet was completed during the experiment to monitor for stress that the animals may have experienced. The mice did not suffer any ill effects during this study. Animal handling, injections and veterinary procedures were strictly conducted by trained South African Veterinary Council (SAVC) registered staff. Animal ethics was reviewed and approved by the University of the Witwatersrand (WITS) Animal Ethics Committee (AEC) (AESC: 2017-010-72B), as well as the National Health Laboratory Service (NHLS) AEC (AESC: 001-19).

### 2.2 *Design and produce recombinant Akirin vaccines*

Primers were designed to amplify the coding region of Akirin (GenBank: KU973613). The Akirin forward primer (5'CACC**ATG**GCGTGCGCAACGTTA3') was 22 bp in length, with a melting temperature of 68 °C and a GC content of 59%. The forward primer was designed to facilitate directional cloning in the pET TOPO® vector (Invitrogen, K101-01), by including the "CACC"

157 overhang sequence, which preceded the initial start codon (ATG) (**bold**). These four nucleotides
158 paired with the overhang sequence, "GTGG", in the pET101/D TOPO® vector. The Akirin reverse
159 primer (5'GGAGAGGTAACTTGGCGCTGCTTC3') was 24 bp in length, with a melting temperature
160 of 67 °C, and a GC content of 58%. The reverse PCR primer was designed to exclude the native stop
161 codon of Akirin, which allowed the PCR product to be cloned in frame with the V5 epitope and the
162 6x His-tag in the pET101/D -TOPO® vector (Invitrogen, K101-01).
163
164 RNA was extracted from 1-day-old female *An. arabiensis* mosquitoes (60 mg), using Trizol™
165 (Sigma Aldrich, T9424). The isolated RNA was purified using the RNeasy® MinElute™ clean-up kit
166 (Qiagen, 74204) following manufacturing protocol. The NanoDrop™ spectrophotometer was used to
167 assess the concentration of the purified RNA, and the integrity of the RNA was assessed using a 1%
168 non-denaturing agarose gel in TBE (Tris/Borate/EDTA), which was electrophoresed for 30 min at 70
169 V.
170
171 The access RT-PCR system (Promega, A1250) was used to reverse-transcribe RNA into cDNA,
172 and amplify the cDNA using the Akirin primers. The RT-PCR reaction contained 1 μM Akirin
173 forward primer, 1 μM Akirin reverse primer, 1 mM $MgSO_4$, 0.2 mM dNTP mix, 0.1 U Avian
174 Myeloblastosis Virus (AMV) reverse transcriptase, and 0.1 U *Thermus flavus* (*Tfl*) DNA polymerase.
175 The sample was made up to a final volume of 50 μl using nuclease-free water and incubated at 45 °C
176 for 45 min, to allow for first-strand cDNA synthesis to take place. The cDNA sample was heated to
177 94 °C, for 2 min, to inactivate the AMV reverse transcriptase enzyme. Once inactivated, 40 PCR cycles
178 were conducted to amplify Akirin (94 °C for 30 sec, 58 °C for 1 min, 68 °C for 2 min), with a final PCR
179 extension at 68 °C for 10 min. The RT-PCR product was purified using the MinElute® PCR
180 purification kit (Qiagen, 28004), and quantified using the NanoDrop™ spectrophotometer. The
181 integrity of the purified RT-PCR product was subsequently assessed using a 1% agarose gel in TAE

(Tris/Acetate/EDTA), which was electrophoresed for 75 min at 100 V, and sent for sequencing (Secugen SL, Madrid, Spain).

The purified RT-PCR product was cloned into the pET101/D -TOPO® vector (Invitrogen, K101-01), using a 0.5:1 molar ratio of RT-PCR product to TOPO® vector. The pET TOPO® construct was transformed into One Shot® TOP10 chemically competent *Escherichia coli (E. coli)* cells (Invitrogen, C6010) using 3 µl of TOPO® cloning reaction. The transformed cells were spread (200 µl) on Lysogeny broth (LB) agar plates, containing 50 µg ampicillin. After an overnight incubation (37 °C), the GenJet™ plasmid miniprep kit (Invitrogen, K0502) was used to isolate and purify the plasmid DNA from selected colonies. Purified plasmids were quantified using the NanoDrop™ spectrophotometer and sent for sequencing.

A sequenced, purified plasmid was transformed into recombinant *E. coli* BL21 Star™ (DE3) One Shot® cells (Invitrogen, C6010-03). The transformation reaction was inoculated into 10 ml of LB media, containing 50 µg/ml ampicillin and 0.5% glucose (CONDA, 21528), and incubated overnight (37 °C, 200 rpm) to propagate cells. After overnight incubation, 1 ml of culture was removed from the propagated LB media, to create a bacterial glycerol stock. The *E. coli* cells, containing the plasmid with recombinant Akirin$^{arabiensis}$, were added to a 50% glycerol solution (1:1) and stored at -80 °C for future use.

Working glycerol stocks of *E. coli* cells containing recombinant Akirin$^{albopictus}$ (GenBank: KU973617.1) and Q38 (GeneBank: JX193856.1) plasmids were used to express these recombinant proteins. These recombinant proteins were initially produced by and maintained at the Instituto de Investigación en Recursos Cinegéticos, IREC, Ciudad Real, Spain [14,29]. *Anopheles arabiensis* Akirin (612 bp) and *Ae. albopictus* Akirin (603 bp) have a sequence similarity of 78%, while *An. arabiensis* Akirin and Q38 (657 bp) have a sequence similarity of 49.1%.

208

209 Using the *E. coli* expression system [34], glycerol stocks were inoculated into 10 ml of LB media,
210 containing 50 μg/ml ampicillin and 0.5% glucose (CONDA, 21528), and were incubated overnight (37
211 °C, 200 rpm) to propagate cells. After overnight incubation, 9 ml of culture was transferred into a
212 flask containing 300 ml of LB media (50 μg/ml ampicillin, 0.5% glucose). The culture was
213 propagated for a further 2-h (37 °C, 200 rpm). IPTG (isopropyl-β-D-1-thiogalactopyranoside) (Sigma-
214 Aldrich, 367-93-1) was added to the propagated culture, to a final concentration of 0.5 mM. The
215 culture was incubated for and additional for 4 to 6-h (37 °C, 200 rpm), to induce heterologous protein
216 expression. Once propagated, the optical density of the culture was measured using the NanoDrop™
217 spectrophotometer, to determine the concentration of bacterial suspension. Centrifugation was
218 conducted to harvest the bacterial cells (3,900 x *g*, 15 min, 4 °C).

219

220 Harvested cells (3 g) were resuspended in 15 ml of lysis buffer (50 mM potassium phosphate,
221 pH 7.8, 400 mM NaCl, 100 mM KCl, 7M Urea, 10 mM Imidazole), which contained a protease
222 inhibitor (Roche, 04693132001). The resuspended cells were mechanically disrupted by sonication
223 (30% amplitude, 30 second on/off pulses), using the Ultrasonic Homogenizer (Bandelin Sonopuls,
224 Model MS73). After sonification, centrifugation was conducted at 15,000 x *g* (15 min, 4 °C). The
225 supernatant, containing the soluble recombinant protein, was transferred to a new tube and the pellet
226 was discarded. The recombinant proteins were purified by nickel affinity chromatography using a 1
227 ml HisTrap™ FF column (GE Healthcare, 11-0012-38 AH), which was mounted on the ÄKTA-FPLC
228 system (GE Healthcare, ÄKTA prime plus). Fractions were eluted in elution buffer (50 mM $KH_2PO_4$
229 pH 7.8, 400 mM NaCl, 100 mM KCl, 7 M Urea, 500 mM Imidazole). Eluted fractions containing the
230 purified protein were collected for dialysis.

231

232 Dialysis was conducted to reduce the salt concentration of the eluted fractions, which contained
233 the purified recombinant proteins. The eluted fractions were transferred into the cellulose dialysis

234 tubing membrane (Sigma-Aldrich, 3110) and incubated in 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM
235 $Na_2HPO_4$, 1.8 mM $KH_2PO_4$ pH 7.4) for 12-h, at 4 ºC. The purified recombinant proteins were
236 quantified using the Pierce™ BCA protein assay kit (Thermo Scientific, 23225), as per the
237 manufacturer's specifications. Total protein quantification was determined using a plate reader (562
238 nm). A sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to assess
239 the integrity of the protein. Total protein (10 μg) was loaded on a 12% SDS-PAGE precast gel (C.B.S.
240 Scientific, BK01212-10), with the Spectra™ multicolor broad range protein ladder (ThermoScientific,
241 26634), and was electrophoresed for 1-h at 180 mA. The gel was stained with Blue BANDit™ (VWR
242 Life Science, K217) and visualized using the gel doc system.
243
244 *2.3 Mouse immunisation*
245
246 The recombinant proteins were concentrated to 375 μg and resuspended to a final volume of 1.5
247 ml using PBS. The recombinant proteins were then emulsified with 1.5 ml of adjuvant (Montanide™,
248 ISA 50 V2) to formulate the recombinant vaccines. A vaccine consisting of PBS and adjuvant was used
249 as a negative control. The mice were vaccinated intraperitoneally, using a double-blind approach,
250 where each treatment was given a code, and the identity of the code was not revealed until the end
251 of the experiment. Therefore, mice were either vaccinated with recombinant protein (25 μg/dose) or
252 the control consisting of PBS and adjuvant. Each mouse received three doses of vaccine, which were
253 administered 2-weeks apart.
254
255 The immunised mice were used to provide female *An. arabiensis* mosquitoes with two
256 subsequent blood meals. Prior to blood feeding, female mosquitoes were mated with male
257 mosquitoes over 4-days, after which the male mosquitoes were removed from the cages. The mated
258 female mosquitoes were 5-days old at the first feeding, and 9-days old at the second feeding.

259 Histamine present in the mosquito's salivary glands did not induce an itch-associated response in
260 mice, thus the mice did not experience discomfort after blood feeding [35].
261
262 However, to prevent discomfort during feeding, the mice were anaesthetised with a mix of
263 Xylazine and Anaket (0.02-0.03 ml). Once anaesthetised, each mouse was placed on top of their
264 respective mosquito cage (BugDorm, 4M1515). Each cage retained a total of 100-mated female
265 mosquitoes, which were starved 12-h prior feeding. The mosquitoes were allowed feed for a total of
266 35 min, after which the mice were removed from the top of each cage. The mosquitoes had a feeding
267 success of approximately 80% overall per treatment. Mosquitoes which did not take a blood meal
268 were subsequently removed from the study after feeding.
269
270 *2.4 Mosquito life-table parameters*
271
272 The rate of survival was monitored until 100% mortality was reached for all four treatments. A
273 Kaplan-Meier survival curve was constructed using Statistix 10. The Log-rank test was used to
274 determine whether there was a statistically significant difference in vector longevity between the
275 treatments ($p < 0.05$) (Additional File 1). Simultaneously, vector fecundity and fertility were also
276 analysed. Two days after receiving the second blood meal, filter paper egg plates were placed at the
277 bottom of each cage. The eggs laid in each plate were counted daily, over a 5-day period. Once the
278 eggs were counted, they were placed into separate plastic containers, filled with 500 ml of $dH_2O$
279 (L:226 mm, x W:166 mm x H:10 mm). The hatchlings were counted over 14-days. Vector fecundity
280 was determined by calculating the mean number of eggs laid per female mosquito, and vector fertility
281 was determined by calculating the mean percentage of hatchlings. A one-way ANOVA test and a
282 Tukey-HSD all pairwise comparison test was used to compare the fecundity and fertility between the
283 treatments ($p < 0.05$) (Additional File 2).
284

285 Vaccine efficiency was used to interpret the overall effect of each antigen on the *An. arabiensis*
286 population tested. Vaccine efficiency is a good method to compare the effect of different antigens on
287 a vector population however, this parameter cannot be compared between vector species, as their
288 developmental processes may differ [5]. Efficiency (E) was calculated considering the effect of
289 vaccination on the reduction of *An. arabiensis* fecundity (Fc), fertility (Fr) and longevity (L) as E (%) =
290 100[(1-L/100)(1-Fc/100)(1-Fr/100)] [16]. A one-way ANOVA test and a Tukey-HSD all pairwise
291 comparison test was used to compare the vaccine efficacy between the treatments ($p < 0.05$)
292 (Additional File 3).
293
294 *2.5 Sample collection*
295
296 ELISA was performed on three blood-fed mosquitoes, to determine whether antibodies, from
297 the vaccinated mice, were ingested by the mosquitoes and if they could be detected. Three engorged
298 mosquitoes were selected from each treatment 24-h post the final feeding. Selected mosquitoes were
299 transferred into separate Eppendorf tubes and stored at -80 °C. Blood samples were also collected
300 from the Saphenous vein of the mice 24-h prior to each immunisation and blood feeding, to determine
301 the antibody titres.
302
303 The mice were warmed prior to blood collection by placing a lamp over the cage for five min.
304 The mice were then placed in a restraining tube, so that the head of the mouse was covered, leaving
305 the hind legs of the mouse exposed [36]. The skin between the tail and the thigh were grasped to
306 obtain the Saphenous vein. Hair was removed from the area using clippers, after which petroleum
307 jelly will be applied to the area to prevent the migration of blood into the surrounding hair. A
308 tourniquet was placed around the leg, above the knee, to allow for puncturing of the vein.
309

The vein was punctured using a 27.5-gauge needle, where drops of blood were subsequently collected. A total of 150 µl of blood was collected from of each mouse, per collection, and dispensed into separate collection tubes. The blood was incubated at room temperature for 30 min to allow for coagulation. Centrifugation was conducted to separate serum from the red blood cells (1,500 x *g*, 10 min, 4 °C). Serum was transferred to new Eppendorf tubes and stored at -80 °C until an indirect ELISA was performed on the samples. Each treatment was repeated in duplicate, using one mouse per treatment. The identity of each treatment was revealed once the last blood sample was completed.

*2.6 Antibody Detection*

An indirect ELISA was performed to assess the antibody titre of each mouse. Recombinant protein was used to coat the 96-well microplate. The recombinant protein (0.1 µg) was diluted in 100 µl tris buffered saline (25 mM Tris HCl, 150 mM NaCl, 2 mM KCl) (TBS), and immobilised to the microplate by overnight incubation (4 °C). After overnight incubation, the microplate was blocked with Blocker™ BLOTTO (ThermoFisher, 37530) (2 h, 37 °C). Blocking solution was aspirated off the microplate. For the quantification of mouse antibodies, the mouse sera samples or the TBS control samples were diluted in blocking solution (1:20, v/v), and hybridised to the microplate (2 h, 37 °C). For the quantification of antibodies taken up by the mosquitoes, the fed mosquitoes were homogenized in 200µl TBS-Tween 20 (TBS-T), and centrifuged to collect the supernatant (3 min, 16,000 × *g*, 4 °C). The supernatant was subsequently hybridised to the microplate (2 h, 37 °C). After hybridisation, the microplate was washed 3-times with TBS-T. Goat anti-mouse IgG antibody, conjugated with horseradish peroxidase (HRP) (Agilent, P0447) was diluted in blocking buffer (1:3,000). The diluted secondary antibody (200 µl) was bound to the primary antibody through a 2-h incubation (37 °C). Unbound secondary antibody was aspirated off the microplate, which was subsequently washed 3-times with TBS-T. SigmaFast™ enzyme substrate (Sigma-Aldrich, N1891) was added to the microplate (200 µl). After a 30-min incubation period, the microplate was analysed

at 450 nm using the multi-well plate reader. The antibody titres were represented as $OD_{450nm}$ ($OD_{mouse\ serum\ or\ mosquito\ sample} - OD_{TBS\ control}$), where sample consisting of TBS were used as a background control. The antibody titres were compared between the treated and control groups, using an unpaired t-test ($p < 0.05$). A Pearson correlation analysis was also conducted in Microsoft Excel to compare the significant effects of antibody response on *An. arabiensis* lifetable parameters ($r < -0.5$).

## 3. Results

### *3.1 Life-table parameters*

The recombinant proteins were successfully produced using the *E. coli* expression system, and emulsified with adjuvant to formulate recombinant vaccines, which were intraperitoneally administered to mice in three separate doses, 2-weeks apart. Engorged female *An. arabiensis* successfully ingested antibodies from the vaccinated mice. Vector longevity was significantly reduced in *An. arabiensis* that ingested blood from mice vaccinated with recombinant proteins, when compared to mosquitoes that ingested blood from the control mice (Log-rank: $\chi^2 = 84$, $p < 0.01$, DF = 3).

*Anopheles arabiensis* which ingested blood from the control mice had a mean survival time of 21-days, while the females which ingested anti-Q38, anti- Akirin$^{albopictus}$, and anti- Akirin$^{arabiensis}$ antibodies had a mean survival time of 16-days, 14-days and 13-days respectively. This was a 24%, 33% and 38% reduction in mean survival time, when compared to the control treatment (Table 1). This is a significant finding as *Anopheles* mosquitoes can transmit malaria sporozoites 14-days post gametocyte infection.

Vector fecundity (One-way ANOVA: $p < 0.001$, $F = 11.7$, $DF = 3$) and fertility (One-way ANOVA: $p < 0.001$, $F = 68.9$, $DF = 3$) were also significantly reduced in *An. arabiensis* which ingested antibodies

362 from mice vaccinated with the recombinant proteins, when compared to the control treatment. The
363 ingestion of anti-Akirin$^{albopictus}$ antibodies reduced vector fecundity by 1.1-fold (42%) and vector
364 fertility by 23%. The ingestion of anti-Akirin$^{arabiensis}$ antibodies reduced vector fecundity by 1.0-fold
365 (50%) and vector fertility by 20%. The ingestion of anti-Q38 antibodies reduced vector fecundity by
366 0.7-fold (53%) and vector fertility by 10%. Overall, the recombinant Q38 antigen had a vaccine efficacy
367 of 67.8% on the targeted *An. arabiensis* vector population, while the recombinant Akirin$^{arabiensis}$ antigen
368 and the recombinant Akirin $^{albopictus}$ antigen had a vaccine efficacy of 73.2% and 72.4% respectively.
369
370 **Table 1.** A comparison of the lifetable parameters between the *An. arabiensis* mosquitoes which
371 ingested blood from the control mice, and the *An. arabiensis* mosquitoes which ingested blood from
372 mice that were vaccinated with recombinant Q38, recombinant Akirin$^{arabiensiss}$ and Akirin $^{albopictus}$
373 proteins
374

| Treatment | Control | Q38 | Akirin$^{arabiensis}$ | Akirin$^{albopictus}$ |
|---|---|---|---|---|
| **Mean survival rate (days) ±SD; N** | 22<br>21<br>20<br>(21 ± 1.0; 190) [a] | 14<br>15<br>18<br>(16 ±1.8; 190) [b] | 12<br>12<br>15<br>(13 ± 1.6; 190) [b] | 15<br>14<br>15<br>(14 ± 0.5; 190) [b] |
| **Fecundity (eggs laid/ total amount of females) ±SD; N** | 68<br>70<br>62<br>(66 ±4.1; 190) [a] | 37<br>22<br>34<br>(31 ±8.1; 190) [b] | 29<br>25<br>46<br>(33 ±11.2; 190) [b] | 28<br>40<br>45<br>38 ±8.9; 190) [b] |
| **Fertility (eggs hatched/eggs laid) ±SD; N** | 0.90<br>0.90<br>0.91<br>(0.91 ±0.01; 190) [a] | 0.83<br>0.81<br>0.81<br>(0.82 ±0.14; 190) [b] | 0.72<br>0.72<br>0.75<br>(0.73 ±0.17; 190) [c] | 0.70<br>0.71<br>0.69<br>0.70 ±0.10; 190) [c] |

| | | | | |
|---|---|---|---|---|
| **Vaccine Efficacy (%)** | - | 67.8 [a] | 73.2 [a] | 72.4 [a] |

375

376 *Results are shown for each vaccine trial (n=3), with the average standard deviation (±S.D.) and total
377 sample size (N). Data was analysed using the Log-rank test and a One-way ANOVA test ($p < 0.05$).
378 Letters "a-c" were used to represent statistical differences between various groups when conducting
379 the Tukey-HSD test.

380

381 *3.2 Antibody detection*

382

383 The antibody titres of the mice that were vaccinated with recombinant protein were significantly
384 higher than that of the control mice. On average, the antibody titres increased 10.4-fold after the first
385 dose of vaccine was administered, an additional 0.5-fold after the second dose was administered, and
386 0.1-fold after the third dose was administered. Overall, the antibody titres of the mice vaccinated with
387 recombinant Q38 were 0.7-fold higher than the antibody titres of the control mice (Unpaired t-test: t
388 = 2.46, $p$ = 0.05, DF = 1). Similarly, the antibody titres of the mice vaccinated with recombinant
389 Akirin$^{arabiensis}$ were 0.9-fold higher than the control mice (Unpaired t-test: t = 2.98, $p$ = 0.03, DF = 1), and
390 the antibody titres of the mice vaccinated with recombinant Akirin$^{albopictus}$ were 0.5-fold higher than
391 the control mice overall (Unpaired t-test: t = 2.90, $p$ = 0.03, DF = 1).

392

393 Females which ingested anti-Q38 antibodies, had an antibody titre which was 1.5-fold higher
394 than the females which ingested blood from the control mice (Unpaired t-test: t = 3.25, $p$ = 0.03, DF =
395 1). *Anopheles arabiensis* females which ingested anti-Akirin$^{arabiensis}$ antibodies had an antibody titre
396 which was 2.6-fold higher than the females which ingested blood from the control mice (Unpaired t-
397 test: t = 7.04, $p < 0.002$, DF = 1), and the *An. arabiensis* which anti-Akirin$^{albopictus}$ antibodies had an
398 antibody titre which was 8.5-fold higher than the females which ingested blood from the control mice

399 (Unpaired t-test: t = 11.92, $p < 0.001$, DF = 1) (Figure 1). The antibody titres of *An. arabiensis* females
400 which ingested anti-Q38 antibodies, anti-Akirin$^{arabiensis}$ antibodies, and anti-Akirin$^{albopictus}$ antibodies
401 negatively correlated with vaccine effects observed on vector survival, fecundity, and fertility.
402
403 **Figure 1.** The antibody response in mice vaccinated with recombinant Akirin proteins, as well
404 as the effect on *An. arabiensis* biology post antibody ingestion. The antibody titres between the
405 recombinant Q38 (blue), Akirin$^{arabiensis}$ (red), Akirin$^{albopictus}$ (green) protein and the control treatment
406 (purple), were assessed 2-weeks after each vaccine dose was administered and 24-h before each blood
407 meal was ingested, at an optical density of 450 nm (a-c). The antibody titres negatively correlated
408 with vaccine effects on vector survival (d-f), fecundity (g-i), and fertility (j-l). The linear correlation
409 coefficients ($R^2$) and Pearson-correlation coefficient are shown (*$r < -0.5$; n = 6).
410

411 **4. Discussion**

412 The significant reduction in vector survival and reproductive capacities characterised by the
413 knockdown of *akirin* in *An. arabiensis* [31], made Akirin a potential antigen for vaccine development
414 against the untargeted outdoor vector populations. Thus, to determine whether Akirin could
415 represent a novel target for the control of *An. arabiensis*, the efficacy of three recombinant vaccines
416 were evaluate, namely recombinant Akirin$^{arabiensis}$, recombinant Akirin$^{albopictus}$, and recombinant Q38.
417 The current data showed no major advantage for the development of a species-specific vaccine
418 however, the impact of dual vaccines still need to be investigated.
419
420 Antibodies against the recombinant proteins were effectively produced in the vaccinated mice
421 and were successfully ingested by the engorged female mosquitoes. *Anopheles arabiensis* longevity
422 was significantly reduced when anti- Akirin$^{arabiensis}$ antibodies, anti- Akirin$^{albopictus}$ antibodies, and anti-
423 Q38 antibodies were ingested. Although not completely understood, it is thought that the ingested

antibodies cross the cell membrane and interact with the target protein in the cytoplasm, thus preventing Akirin from translocation to the nucleus [8,37].

After ingesting a bloodmeal, the mosquito's midgut bacterial levels increase rapidly [38]. An increase in midgut bacterial levels causes an increase in bacterial elicitors, such as peptidoglycan (PGN) [39]. The IMD pathway is activated when PGN binds to a transmembrane peptidoglycan recognition protein known as PGRP-LC [40]. PGN induces a conformational change when binding to PGRP-LC, causing DREDD to translocate to the plasma membrane from the nucleus [41]. DREDD-mediated cleavage of Relish [42], allows Relish to translocate into the nucleus [43].

In the nucleus, Relish is regulated through interactions with Akirin and the BAP60 component of the Brahma (SWI/SNF) chromatin remodelling complex [21]. Akirin and the BAP60 form a complex, which binds to Relish and forms a link on the promoter of a subset of NF-kB target genes [22]. This allows for the gene expression of several antimicrobial peptide-coding genes, such as Attacin and Diptericin [44-46]. The antimicrobial peptides subsequently reduce the number of microbiotas in the midgut to the basal level [44,45]. Targeting Akirin therefore disrupts bacterial homeostasis and weakens the innate immune defence required for survival [21,38,47].

Mosquito longevity is a critical factor with regards to malaria transmission, as the female must survive longer than the extrinsic incubation period (EIP) to transmit malaria [48]. To transmit malaria, a female *Anopheles* mosquito must ingest a blood meal infected with gametocytes. The ingested parasites must encounter the midgut epithelium and develop into male microgametes and female macrogametes. The gametes must undergo fertilization to form zygotes and mature into motile ookinetes and transverse across the midgut epithelium [49]. Ookinetes which evade the mosquito's immune responses must develop into oocysts and produce haploid sporozoites. The sporozoites

449 must be released into the mosquito hemocoel, migrate through the mosquito haemolymph, and
450 invade the salivary glands, to complete the sporogonic cycle [50].
451
452 It takes approximately 14-days to complete the sporogonic cycle. Once complete, the sporozoites
453 are subsequently injected into the next vertebrate host when taking a successive blood meal [51,52].
454 This allows for the transmission of malaria. Eliminating a third of the vector population prior to the
455 completion of this incubation period, could significantly affect the transmission of malaria.
456 Additionally, both vector fecundity and vector fertility were significantly reduced when anti-
457 Akirin$^{arabiensis}$ antibodies, anti- Akirin$^{albopictus}$ antibodies, and anti-Q38 antibodies were ingested by *An.*
458 *arabiensis*.
459
460 It has been postulated that the movement of antibodies to reproductive tissues interferes with
461 oogenesis, as the synthesis of vitellogenin by the fat bodies as well as its uptake of vitellogenin from
462 the haemolymph may be inhibited [53]. Alternatively, it has been suggested that the ingested
463 mosquito antibodies may irritate the gut, which in turn reduces the blood meal or the availability of
464 nutrients, thus the content of some of the developing follicles may be reabsorbed, resulting in reduced
465 vector ovipositioning [53]. Akirin, however, is known to play a critical role in processes unrelated to
466 immunity, such as development and reproduction [14,21]. Regardless of the underlaying mechanism,
467 reducing the amount of eggs laid by half, as well as the amount of viable progeny by a fifth could
468 reduce the *An. arabiensis* population.
469
470 Mosquitoes that have taken an infectious blood meal usually take three to four additional blood
471 meals before completing the sporogonic period. This would ensure repeated ingestion of anti-
472 mosquito antibodies, which could disrupt gut bacterial homeostasis, ultimately reducing the vectors
473 lifespan [38], as well as reproductive fitness. Reducing *An. arabiensis* survivorship as well as

474 reproductive fitness would ultimately cause a reduction in vector population abundance. A reduction
475 in vector abundance would have a significant impact on malaria transmission.
476
477 The reduction of these *An. arabiensis* lifetable parameters after the ingestion of anti-Akirin$^{albopictus}$
478 antibodies was an interesting observation, as previous recombinant Akirin$^{albopictus}$ immunisation
479 experiments affected *Ae. aegypti*, *Ae. albopictus*, and *An. coluzzii* oviposition, but not longevity [27,28].
480 These differences were attributed to the physiological difference between the various genera and
481 species, as these results may vary based on the amount of protein ingested by each vector species, the
482 difference in antibody titres of the vaccinated hosts, as well as the biological activities of the
483 mosquitoes, which are influenced by sensitivity to proteases within the midgut [54].
484
485 Another possible reason for the variability of these results could be due to the system used to
486 make these recombinant vaccines. In previous immunisation studies, the *Pichia pastoris* expression
487 system was used to express recombinant Akirin$^{albopictus}$ and recombinant Q38 antigens [27-29], while
488 this investigation used the *E. coli* expression system. One way to overcome this would be to
489 standardize mosquito vaccine research, similar to that proposed for tick vaccines [55], to allow for
490 the comparison of results between different research groups These parameters would need to include
491 the standardisation of the expression system used, the type of adjuvant, the experimental procedures
492 followed to determine the antibody titres and vaccine efficacy, the sample size used to make these
493 comparisons, as well the age of the experimental animals used within the study.
494
495 When comparing the efficacy between the three recombinant vaccines, the difference was
496 negligible. However, the chimeric nature of Q38 makes this antigen a valuable multi-target arthropod
497 vaccine. The efficacy of Q38 has been previously tested on *Ixodes ricinus* and *Dermacentor reticulastus*
498 (ticks) as well as *Phlebotomus perniciosus* (sand flies) [29,56]. The ingestion of anti-Q38 antibodies
499 significantly reduced *I. ricinus*, longevity by 4.25-fold as well as moulting by 38%. The ingestion of

anti-Q38 antibodies significantly reduced *D. reticulastus* mass by 25% as well as moulting by 15% [56]. When anti-Q38 antibodies were ingested by *P. perniciosus,* oviposition was reduced by 16–26% [29].

The conserved structure and function of Akirin throughout the metazoan [17,25] has, however, risen the question of safety when vaccinating vertebrates. As in previous experiments with different hosts, physiological or pathological alterations were not observed in vaccinated mice [14]. This suggests that as expected, the antibody response was directed against non-self-epitopes, thus reducing the possibility of detrimental effects to the vaccinated host. Furthermore, Akirin is an intracellular antigen and for still unknown mechanisms antibody can enter arthropod but not mammalian cells [8]. Additionally, effective immunisation of various hosts, for the control of numerous vector infestations, displays the low risk of inducing an autoimmune response in vertebrates [14,27,29].

Challenging various blood hosts with whole protein or antigenic peptides may provide a valuable intervention against several ectoparasites including the untargeted exophagic and exophilic *An. arabiensis* mosquito populations. This is a valuable intervention for the control of *An. arabiensis,* as the human malaria parasites are unable to amplify within these reservoir hosts. In future, additional studies will be essential to vaccinate agricultural animals with recombinant Akirin antigens to evaluate the impact on *An. arabiensis* wild populations.

## 5. Conclusions

The results reported here were the first showing that hosts vaccinated with recombinant Akirin$^{arabiensis}$ can develop a protective response against mosquito populations. Hosts vaccinated with recombinant Akirin$^{albopictus}$ and recombinant Q38 also developed a protective response against *An. arabiensis*, and thus existing vaccines could be used to target this vector population. The reduction in

*An. arabiensis* survival and reproductive capacities, due to revivor-host immunisation, has provided a step toward the development of a novel target for the control of *An. arabiensis.*

## 6. Declarations

**Ethics:** Animal ethics was reviewed and approved by the University of the Witwatersrand (WITS) Animal Ethics Committee (AEC) (AESC: 2017-010-72B), as well as the National Health Laboratory Service (NHLS) AEC (AESC: 001-19).

**Consent for Publication:** Not applicable.

**Availability of data and materials:** Additional File 1: Data for survival analysis. Additional File 2: Fecundity and fertility dataset. Additional File 3: Calculation for vaccine efficacy

**Competing interests:** The authors declare no competing interest.

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**Author Contributions:**

Conceptualization, L.L.K.; methodology, B.D.L.; L.L.K.; Y.D.; M.C.; J.d.l.F. ; software, B.D.L.; M.C.; validation, L.L.K.; Y.D.; M.C.; J.d.l.F.; formal analysis, B.D.L.; M.C.; L.L.K.; investigation, B.D.L.; I.L.; resources, L.L.K.; J.d.l.F.; data curation, B.D.L.; writing—original draft preparation, B.D.L.; writing—review and editing, L.L.K.; Y.D.; M.C.; J.d.l.F.; I.L.; visualization, B.D.L.; Y.D.; L.L.K.; supervision, L.L.K.; Y.D.; M.C.; J.d.l.F.; project administration, L.L.K.; funding acquisition, L.L.K.; Y.D.; J.d.l.F. All authors have read and agreed to the published version of the manuscript.

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# APPENDIX G

- Plagiarism Declaration
- Supervisor’s Acknowledgment and Motivation Letter
- Turnitin Report

**PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS**

SENATE PLAGIARISM POLICY:

I, Blaženka Danica Letinić (Student number:569608), am a student registered for the degree Doctor of Philosophy in the academic year 2020.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature:  Date: 8 August 2020

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4 August 2020

To whom it may concern,

**Turnitin Report**

The Turnitin report generated a total of 190 sources, which displayed a total similarity index of 28% to the text presented in this thesis. Upon further analysis of the report, 12% of the similarity index was due to a manuscript which was generated from this research and was submitted to PloS one for publication (The PhD student is the first author on these publications). All subsequent sections used from the manuscript have been referenced.

A similarity index of approximately 7.82% was generated from 99 of the Turnitin sources identified. This was due to various general scientific techniques used to conduct the experiments presented in this thesis, some of which were published from this thesis. The methods used were adapted from the instructions supplied by the manufacturers for the kits used, or from published literature which was referenced. A total similarity index of 2.03% was due to general information about malaria and the vectors which transmit the *Plasmodium* parasites (14 Turnitin sources).

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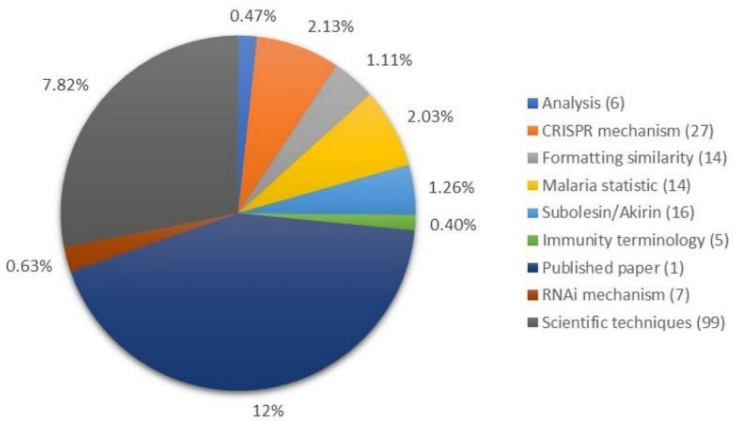


We hope you find the explanation informative, but please do not hesitate to contact us, should you require further information.

Yours sincerely,

| | | |
|---|---|---|
| Prof Lizette L. Koekemoer (PhD,FRES)<br>Supervisor<br>Co-Director, Wits Research Instititue for Malaria<br>NRF/SARChI Chair: Medical Entomology and Vector Control<br>University of the Witwatersrand | Dr. Yael L. Dahan-Moss (PhD)<br>Supervisor<br>Senior medical scientist at Vector Control Reference Laboratory, National Institute for Communicable Diseases.<br>Joint Lecturer with the Wits Research Institute for Malaria, University of the Witwatersrand. | |

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