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Magnetoreception in Robins

Radboud Honours Proposal

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Finally we thank all other Honours students for their input during the introduction and progress meetings.

[1] An experiment which we proposed, but which did not make it as our final proposal.

Contents

1. Basic Details

1.1. Abstract

Even with the current scientific advances in biology, much remains unclear about the underlying mechanism of magnetoreception of migrating European robins, *Erithacus rubecula*. One of the major hypotheses regarding this topic postulates a magnetically sensitive radical pair reaction in photoreceptors in the eyes, induced by light, serving as a foundation for the bird's ability to sense information about the Earth's magnetic field. The cryptochrome protein is currently the only candidate to contain this radical pair mechanism. It is known that robins possess four distinct types of cryptochrome (1a, 1b, 2 and 4), but there is no consensus on which one would be responsible for magnetoreception. The aim of the proposed project is to establish whether cryptochrome is involved in the magnetoreception, and if this is the case, which type. This will be accomplished by genetic modification and behavioural experiments. Using AAV-mediated CRISPR-Cas9, the different types of cryptochrome, each separately as well as all four at once, will be knocked out in the retina of the robins. The birds' migrating abilities after the knockout will be assessed in behavioural experiments to determine which cryptochrome, if any, is necessary for magnetoreception and migration. This project would be a step forward in the mysterious phenomenon of bird migration. Furthermore, this would open the array of gene editing techniques to a new species.

1.2. Summary

Despite the general acceptance of the fact that European robins use the Earth's magnetic field to navigate during the migratory seasons, the mechanism behind the magnetic sensing of European robins is not yet fully understood. One of the most promising mechanisms has a quantum mechanical foundation. Herein, two electrons interact with the magnetic field, which causes a change in configuration. This change in configuration then leads to a signal which encodes the magnetic field information. This system can very well be embedded in the so-called cryptochrome protein, which comes in four different types. However, there is no direct experimental evidence that any of these types of cryptochrome is involved in the magnetoreception of European robins. Therefore, we propose an innovative experiment to test the hypothesis of the involvement of cryptochrome in the magnetic sensing ability of European robins. We intend to do this by first applying *in vivo* gene editing techniques to the retina, to disable the different types of cryptochrome individually as well as all four types at once. We then perform behavioural tests for the magnetic sensing of the birds in a specialised facility. Succeeding with this experiment would be a major step forward in the understanding of the magnetic sensing of European robins. Furthermore, this would open the array of gene editing techniques to a new species.

1.3. Samenvatting

Hoewel het algemeen geaccepteerd is dat roodborstjes het aardmagnetisch veld gebruiken om te navigeren tijdens de migratieseizoenen, is het mechanisme waarmee het roodborstje het aardmagnetisch veld voelt niet bekend. Het meest waarschijnlijke mechanisme gebruikt een quantum mechanisch effect, waarbij twee elektronen reageren op het magnetisch veld, en daardoor van configuratie veranderen. Deze verandering bevat de informatie over het aardmagnetisch veld. Dit systeem van twee elektronen bevindt zich waarschijnlijk in een zogenaamd cryptochrome eiwit. Er zijn vier verschillende types van dit eiwit, maar het is nog nooit experimenteel aangetoond dat een van de cryptochrome eiwitten daadwerkelijk betrokken is bij het voelen van het magneetveld. Daarom stellen wij een innovatief experiment voor om deze hypothese van de betrokkenheid van cryptochrome te testen. Onze intentie is dit te doen door eerst door genetische manipulatie *in vivo* in het oog van de roodborstjes de verschillende soorten cryptochrome uit te schakelen. Daarna wordt de magnetoreceptie van de roodborstjes in een gedragsexperiment in een speciale faciliteit getest. Wanneer dit slaagt is dit een grote stap richting het begrijpen van het mechanisme achter het voelen van het magneetveld door roodborstjes. Daarnaast opent dit de methodes van genetische manipulatie voor een nieuwe diersoort.

1.4. Keywords

avian magnetoreception; cryptochrome; robins; radical pair mechanism; in vivo gene knockout; CRISPR-Cas9; AAV

2. Introduction

For half a century, it has been known that European robins, *Erithacus rubecula*, use the Earth's magnetic field for migratory orientation [84]. Still, the mechanism for this magnetoreception is not fully understood [24, 36, 40].

There are two main hypotheses for magnetoreception. One involves magnetite particles in the upper beak [12, 32]. The other involves a light-dependent radical pair mechanism (RPM) in the eye [61, 63, 66].

While the first mechanism has been questioned [45, 74, 75, 91], the second mechanism has quite some experimental evidence. This includes the following observations:

1. In vitro RPMs have been shown to be sensitive to Earth strength magnetic fields [30, 39]. This founds the possibility of a RPM.

2. Magnetoreception is light-dependent, and depends on the wavelength of this light [48, 85]. This is expected for a RPM to form the radical pair [24].

3. Pulse remagnetisation is absent, which would be expected in case of magnetoreception through magnetite particles [23].

4. Magnetoreception is disrupted by weak broadband electromagnetic fields [67]. While this effect is not predicted by current RPM models, it is very unlikely to be caused by magnetite particle magnetoreception [22].

In their 2000 paper, Ritz et al. [61] suggest that cryptochrome might be part of the magnetoreception system. In fact, cryptochrome remains the only known candidate for magnetoreception through a RPM to date [20, 24, 36, 47].

Moreover, there is some, but mainly circumstantial, experimental evidence for involvement of cryptochrome in magnetoreception.

1. Gegear, Casselman, et al. [16] and subsequent papers [13, 17] claim to show cryptochrome dependent magnetoreception in fruit flies, *Drosophila melanogaster*. However, in an attempt to reproduce these results, Reichl et al. [60], using much larger sample sizes, could not reproduce any magnetoreception in *Drosophila melanogaster*.

2. Cryptochrome 1a occurs in structures which could immobilise and align the molecules [53].

3. Cryptochrome 4 is expressed at a constant level throughout the day, suggesting it is not part of the circadian clock [20, 55].

The papers referenced in the first item are the only papers providing direct evidence for the involvement of cryptochrome in the magnetoreception [24, 60]. The irreproducibility of these findings brings with it a major need for direct experimental evidence for cryptochrome's involvement in the magnetoreception. The intent of the proposed project is to fulfil this need, and at the same answer the long-debated question of *which* type of cryptochrome, if any, is involved in the magnetoreception.

3. Project Description

As it has been pointed out in the previous section, the current experimental evidence for the involvement of cryptochrome is either irreproducible or circumstantial. Therefore the aim of this project is to provide direct experimental evidence for the involvement of cryptochrome. This will be done by first applying novel gene editing techniques *in vivo* to disable each type of cryptochrome individually as well as all at once. This knockout will be performed locally in the retinas of the European robins using AAV vectors. Then behavioural tests will be performed with these genetically modified European robins to test their ability to use magnetic fields in navigating. Significant effort is put in establishing the fact that observed effects are actually caused by the absence of cryptochrome. To this end, the robins' motivation to migrate is, for example, tested behaviourally as well. Finally, the data obtained through these behavioural experiments will be analysed to establish whether the European robins need the cryptochrome protein

to use their magnetic sensing ability. Although the behavioural tests are a well-established manner for testing migratory behaviour of the European robins, the *in vivo* gene knockout has not been attempted before in European robins.

In the subsequent sections the background information for the different aspects of the current project and the methods for each step will be covered.

4. Background

This part of our proposal serves the purpose of providing contextual background information to our proposed experiments. It consists of four main sections, the first one describing the habitat, distribution and migratory patterns of European robins. Next, we will discuss three levels of the magnetoreception theory that we are testing: The quantum mechanical level, the chemical level and the biological level. The quantum mechanical level involves the radical pair theory, which serves as a basis for the hypothesis. The biological level concerns the cryptochrome protein, where the radical pair mechanism is thought to be formed. The chemical level discusses the chemical pathway that is present in cryptochrome, the connection between biology and quantum physics. The last section delves into the aspect of gene editing. There you can find an explanation of the gene-modification technique we will use, CRISPR-Cas9, and information about adeno-associated viruses, which we will use as a vector for the delivery of CRISPR-Cas9 in the European robins.

4.1. European Robins, *Erithacus rubecula*

Figure 1: An adult robin [14].

The European robin, *Erithacus rubecula* is a small insectivorous bird from the large

family of Old World flycatchers, *Muscicapidae*. They are widely distributed in Europe, the United Kingdom, Russia, and the Middle East, see figure 2. Certain populations of robins are migratory and even fly to Northern Africa for wintering [2]. Robins in Scandinavia and Russia migrate to the UK and Western Europe during harsh winters. It is generally thought that Eastern populations of robins are migratory, whereas Western populations are mostly resident [8].

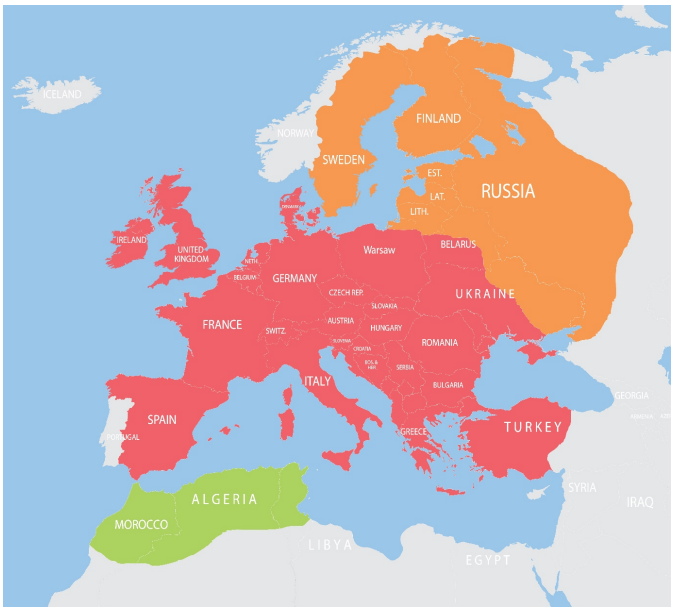

Figure 2: The distribution of Erithacus rubecula in Europe. Orange area represents Summer range, red area — residence range, green area — wintering range [76].

Adult robins are between 12.5 cm and 14.0 cm in length and weigh 16 g to 22 g. Male and female individuals are similar in colouration, with a trademark orange breast. Juveniles do not exhibit this colouration. They have a spotted brown and white colouration [25].

Their lifespan is 3 to 5 years. During mating season from April to August, robins usually lay 4 to 6 eggs in nests in bushes and hedges.

Robins are diurnal species. They feed on terrestrial invertebrates such as earthworms when available. In autumn and winter, resident robins eat spiders, worms, berries and fruit [25].

4.2. Radical Pair Mechanism

The proposed mechanism for the magnetoreception of robins on the molecular level is the so-called radical pair mechanism (RPM) [24, 40, 61]. The name of this mechanism is derived from the fact that there is a pair of radicals in the chemical systems which form

an entangled quantum system. A radical is a molecule with an unpaired electron. In the following sections, we will briefly discuss this mechanism from a quantum mechanical point of view, and then turn to the possible embedding of the radical pair mechanism in the cryptochrome protein, present in the robin's retina.

Chemically, the radical pair mechanism has several steps, see figure 3. It starts with a pair of molecules, the donor (D) and the acceptor (A). When light is incident on the system, a radical pair is formed, with an electron transferring from the donor to the acceptor. This forms a radical pair which can interact with a magnetic field as will be described below. This radical pair can be either in a singlet state in which the electron spins are unaligned or in a triplet state where the electron spins are aligned. Either a singlet product (SP) or a triplet product (TP) will form based on this state [62].

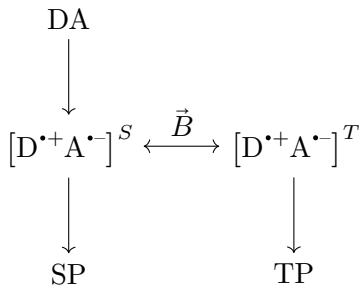

Figure 3: General radical pair mechanism

The quantum mechanical system consists of two electrons and two molecules, viewed as nuclei, see figure 4. In general, the electrons interact with their corresponding molecule, each other and the external magnetic field. Here, we consider a simplified model in which there is no kinetic energy term and only the Zeeman interaction and the hyperfine interaction are taken into account.

The Zeeman interaction is the interaction between the electron spin and an external magnetic field. The spin of the electron associates a magnetic moment with the electron, and through this the spin of the electron is influenced by the external magnetic field.

The hyperfine interaction stems from the interaction between the electron and the molecule it is bound to. The molecule has a net spin as well and this interacts with the spin of the electron. For dynamic behaviour this interaction has to be anisotropic, otherwise the system will be invariant under a change of magnetic field direction, i.e. one can always arrange the direction to be along the z-axis. This anisotropy is realised because the molecules that form a radical pair are not spherically symmetric [69].

Simulations of this system, see appendix C for a more detailed description, show that the probability of finding the electrons in a singlet state changes over time depending on the direction of the magnetic field, see figure 5. This can be seen from the fact that the amplitude, in the case that the magnetic field is perpendicular to the z-axis, decreases

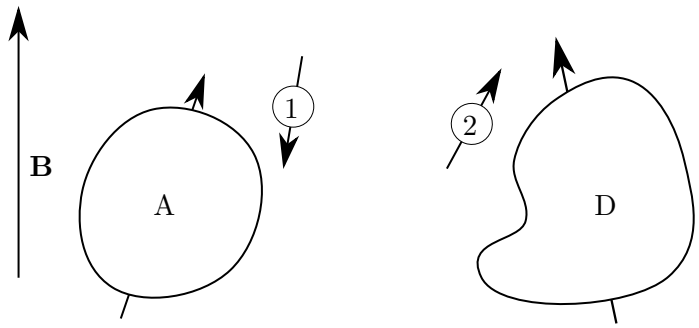

Figure 4: Schematic drawing of a radical pair system, with donor and acceptor molecules (D & A) and two unpaired electrons (1 & 2).

over time, whereas it remains the same in the case that the magnetic field is aligned with the z-axis. This shows that a compass that relies on the radical pair mechanism could exists, from a quantum mechanical point of view.

More complicated models take other interactions into account, such as the exchange interaction and dipolar interaction. Based on theses models one finds a low-field effect, which could explain the magnetic sensing abilities of the robins [24, 62].

The radical pair mechanism is likely to occur in the eye because it is light-dependent [82, 85]. As we will see in the next section, it could very well be embedded into the protein cryptochrome, with cofactors FADH and tryptophans [24, 55, 69, 71]. However, superoxide, an oxygen ion, is also named as a possible molecule for the radical pair [70].

4.3. Cryptochrome as a Magnetoreceptor

Cryptochromes are a large group of light-sensitive proteins present in numerous living organisms, from prokaryotes (bacteria) to plants and animals. They are mostly involved in growth regulation in plants and circadian rhythms of plants and animals. In recent years, cryptochrome has been proposed to be responsible for avian magnetoreception [37, 61].

Cryptochromes are 50 kDa to 70 kDa blue-light photoreceptor flavoproteins, enzymes involved in respiration and photosynthesis, see figure 6. They contain 2 non-convalently bound chromophores, light-absorbing molecules: A redox-active flavin adenine dinucleotide (FAD; a strong electron carrier) and a light-harvesting cofactor, able to transfer light energy. Furthermore, cryptochrome contains three tryptophan molecules [49].

Robins possess three distinct types of cryptochromes: Cry1 of which two isoforms, Cry1a and Cry1b are present, Cry2, and Cry4b. There is still an ongoing debate about which of the four cryptochromes is involved in magnetoreception of robins.

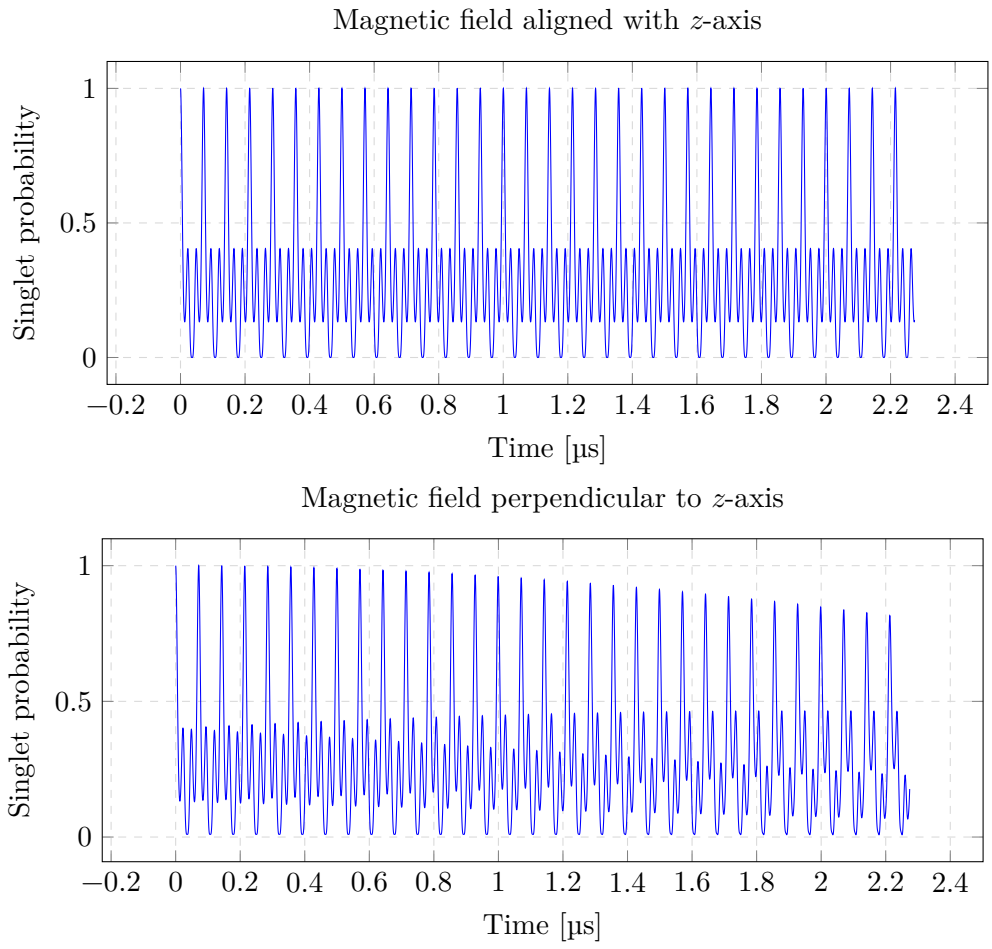

Figure 5: Results of the simulation. The hyperfine interaction only had an x- and y-component. The magnetic field was of geomagnetic strength. The hyperfine interaction was 20 times stronger.

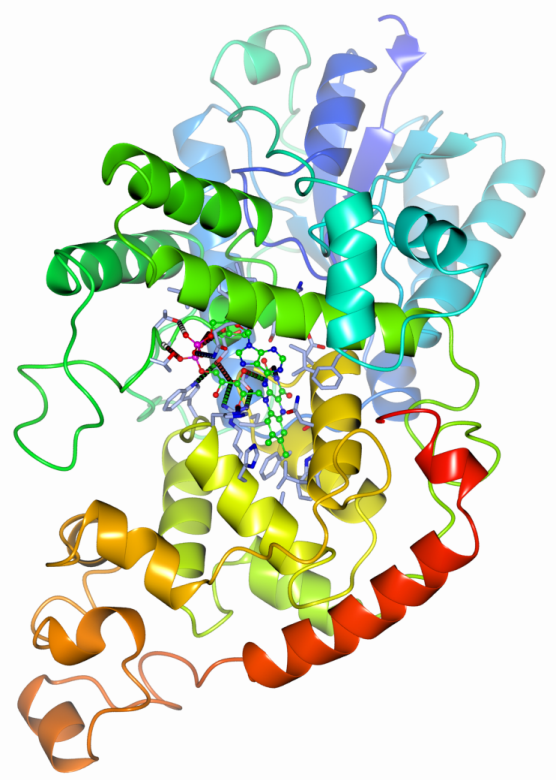

Figure 6: Cryptochrome 4 structure. Rendered using the data from SWISS-MODEL [6].

Cry1a is expressed in the cytoplasm of UV/V cones in the retinas of the birds. Cry1b is expressed in the ganglion cells in the eye and the brain. Cry1 expression is rhythmic and is expressed more during the light phase of circadian clock, and less during the circadian night. But when examined in chickens, the light did not have much influence on Cry1 expression, so it may not be light-sensitive and not involved in migratory birds, because normal chickens and other non-migratory birds also have it. Additionally, some studies are suggesting Cry1 also cannot bind FAD *in vivo* [49]. Cry2 is found in the nucleus of the cells of the photoreceptor layer. Cry4, located in retinal pigment epithelium (below the rods and cones), binds to FAD and is the most likely candidate for magnetoreception in robins [20, 55].

However, another study where they looked into European robins and domestic chickens, observed different results [53]. They suggest Cry1 as the protein involved in magnetoreception. It is located in cytoplasm, but can be fixed along the membrane in the outer segment of UV/V cones in the retina.

4.3.1. Chemical Pathway of Cryptochrome Activation

After cryptochrome absorbs blue light, a protein-bound flavin and three tryptophan (tyrosine) radicals are formed. The activation mechanism described below is one the proposed theories of how cryptochrome is activated and how it could potentially be involved in circadian clock regulations and magnetoreception in plants and animals [20]. This mechanism is also schematically represented in figure 7.

1. The reaction is initiated when FAD absorbs a photon, turning into an excited state FAD^*, which becomes protonated from a nearby aspartic acid. FAD^* becomes $FADH^+$.

2. An electron jumps from Trp400 to $FADH^+$, in a hole left by the excited electron.

3. FADH and $Trp400^+$ are the first radical pair. The reduced FADH and Trp400 both have an unpaired electron, with spins in a correlated state, either singlet or triplet state.

4. FADH and $Trp377^+$ are second radical pair. An electron from Trp377 jumps to Trp400, and Trp377 becomes protonated. Trp400 is reduced, and returns to its ground state.

5. FADH and $Trp324^+$ form third radical pair. An electron from Trp324 jumps to Trp377.

6. Trp324 becomes deprotonated, which also possible for the electron to back-transfer to FADH, if radical pair is in singlet state.

7. FADH oxidises back to FAD under aerobic conditions and cryptochrome becomes inactive.

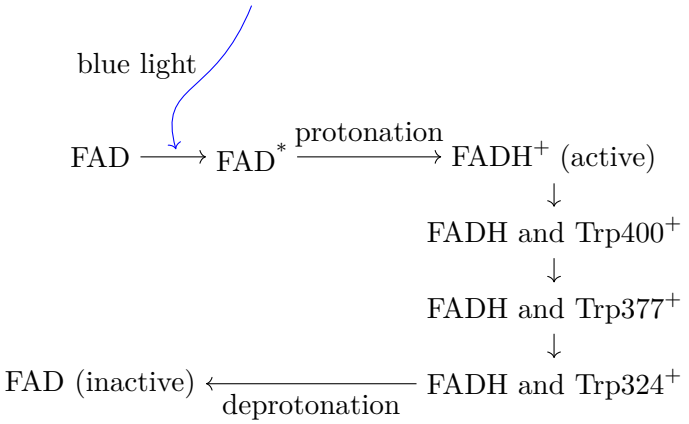

Figure 7: The hypothesised pathway of cryptochrome activation.

The singlet-triplet state interconversion influences how long a cryptochrome spends in its active state [61, 69, 70].

4.4. AAV-Mediated CRISPR-Cas

In this section, the gene editing technique we will use, CRISPR-Cas9, and the vector we will use to deliver it, adeno-associated virus (AAV), are described. First the CRISPR-Cas9 system will be described. Then the adeno-associated virus will be described. The third section will describe how AAV can be used as a viral vector. Finally, the last section will describe how CRISPR-Cas9 specifically can be delivered using AAV.

4.4.1. CRISPR-Cas9

CRISPR-Cas is an adaptive immune system that uses RNA-guided nucleases (e.g. Cas9) to cleave (or cut) foreign genetic elements. The system was first discovered in bacteria which use it as an immune system to fight viruses [28, 59]. Upon invasion, the CRISPR-associated nuclease attaches to a single guide RNA (sgRNA) which guides the nuclease to the invading DNA sequence called the "protospacer" sequence. All protospacer sequences carry a protospacer adjacent motif (PAM), a 2 to 6 basepair (bp) DNA sequence present in the invading virus, but not present in the host genome. This makes it an essential component for the targeting by the nuclease; the nuclease will only cleave a sequence if there is a PAM present in the sequence [28].

When used for genome modification purposes, the sgRNA can be altered in order to target a specific genetic sequence [59]. After cleavage by the nuclease, the targeted

sequence tries to repair itself through one of two pathways: the error prone nonhomogolous end joining (NHEJ) or the high-fidelity homology directed repair (HDR) [59]. NHEJ can be used to mediate gene knockouts, since insertion and deletion mutations (indels) occurring within a coding part of the DNA or RNA can stop the DNA or RNA from functioning. HDR can be used for precise modifications in the sequence, but this repair pathway is only activated when a "repair template" is introduced as well [59].

There are multiple types of CRISPR systems. Type II is one of the best characterised, which uses the Cas9 nuclease to cleave the targeted DNA [28, 59]. The main difference between these types of CRISPR systems is the nuclease used to cleave the DNA. Depending on this nuclease, a different PAM is used [59].

4.4.2. Adeno-Associated Virus

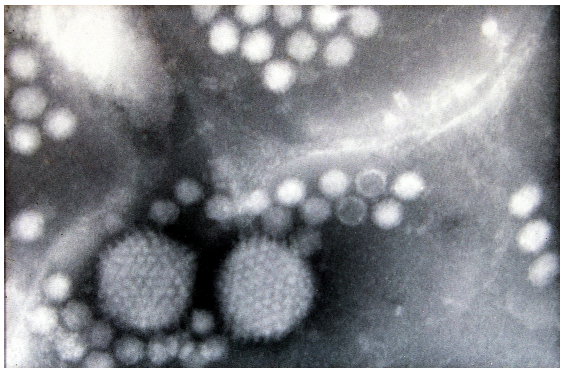

Figure 8: Two adenovirus particles surrounded by numerous, smaller adeno-associated viruses (negative-staining electron microscopy, magnification approximately 200 000 *times) [5].*

The adeno-associated virus (AAV) can often be found in the neighbourhood of adenoviruses, see figure 8, and is constructed based on a single stranded DNA (ssDNA) of about 4.7 kilobase (kb) length [80]. It has an inverted terminal repeat (ITR) at both ends of the DNA strand and it has two open reading frames (ORFs): Rep and Cap. Rep is the protein involved with the AAV genome replication and the packaging into the capsid[2]. Cap is the protein that forms the capsid. Additionally, AAP, an accessory protein, which is not encoded in the ssDNA, is needed for the AAV production [18]. Figure 9 shows the basic structure of the AAV.

4.4.3. AAV as Viral Vector

Viral vectors are tools commonly used in molecular biology for gene delivery [80]. AAV could be used as viral vector for genetic modification by removing the Rep and Cap from

[2]A capsid is the protein coat of a virus particle, surrounding the nucleic acid or nucleoprotein core.

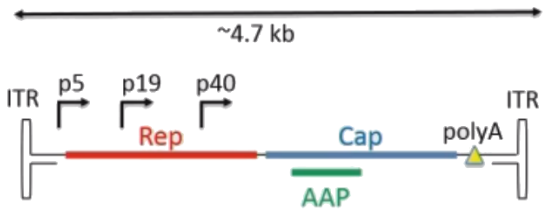

Figure 9: Basic structure of AAV [18].

the DNA of the AAV and subsequently inserting the desired gene between the ITRs [19]. However, there is a major drawback: the limited length of the AAV. The AAV has a small genome size (4.7 kb), thereby restricting the size of the gene that can be inserted in the viral vector.

There are eleven AAV serotypes (AAV1, AAV2, ..., AAV11) of which AAV2 is most extensively studied [80]. The serotypes each differ in tropism.[3] AAV2, AAV8 and AAV9 lead to a high transduction in retinal cells [7, 54, 88].

4.4.4. CRISPR-Cas9 Delivery

As described in the previous section, AAV could be used as viral vector for genetic modification by removing the Rep and Cap from the DNA of the AAV and subsequently inserting the desired gene between the ITRs [19]. The Rep and Cap can be replaced with a CRISPR nuclease gene (e.g. a Cas9 gene), together with a sgRNA, to integrate the CRISPR-Cas9 machinery into the AAV.

Streptococcus pyogenes Cas9 (SpCas9) is the most commonly used CRISPR nuclease, with PAM sequence NGG where N is any nucleotide [77]. However, its drawback is the 4 kb coding sequence it has, making it impossible to package the necessary gene constructs into a single AAV vector [77]. To solve this problem, *Staphylococcus aureus* Cas9 (SaCas9) could be used, which is 1 kb shorter than SpCas9. Its PAM sequence is NNGRRT where N is any nucleotide and R is either A or G. Another option is to create a dual-vector AAV system where SpCas9 goes into one vector and the sgRNA and possible markers go into the other vector, together composing the CRISPR machinery needed [26, 90].

5. Method

In this section the design of each experimental step will be discussed. The first section describes the methodology concerning the cryptochrome gene knockout. The experimental setup for AAV-mediated CRISPR-Cas9 system, first *in vitro* on cell cultures and then

[3] Tropism is the preference for a specific tissue.

on birds *in vivo* will be discussed, together with methods for confirming the knockout. The next section deals with the behavioural experiments that follow the gene knockout. These experiments cover both the motivation to migrate and the magnetoreception. The last section deals with the statistical analysis of the experimental results.

5.1. Gene Knockout

We plan to perform a knockout of the different types of cryptochrome that are naturally found in European robins, *Erithacus rubecula*. We intend to do this by using *in vivo* AAV-mediated CRISPR-Cas9. In order to maximise the potential for success, we propose several possibilities for this, which will be conducted in parallel. The first option is a two-component system, where one AAV vector contains the SpCas9 gene, and one AAV vector containing the code for guide RNA and a fluorescent marker, as it has been done before in mice [26, 90]. The second option is a single AAV vector with a shorter Cas9 orthologue, SaCas9, which allows for the genetic information for the guide RNA (gRNA) to be inserted into the same plasmid without exceeding the capacity of the AAV capsid [58].

5.1.1. Plasmid Development

Plasmid development is a significant part of gene modification, since it is the foundation for the subsequent steps. A natural plasmid is a small circular DNA found in bacteria that replicates separately from chromosomal DNA. It is often used in genetic manipulation experiments since it can be modified easily. In our case, the plasmids will contain the "package" of sequences that will be inserted in the AAV — Cas9 sequence, CRISPR sequence and a marker. This section treats the detailed information about plasmid design.

In general, a CRISPR plasmid must contain:

U6 promoter A gene that drives the expression of the gRNA and reduces off-target effects [35].

guide RNA (gRNA) In itself a synthetic fusion of two bacterial RNAs that provide both targeting specificity and binding ability for the Cas9 nuclease, approx. 100 bp in length [65].

- **crisprRNA (crRNA)** An approx. 20 nucleotide spacer sequence that guides RNA to a specific target location in the genome.
- **tracrRNA (tRNA)** A scaffold sequence, responsible for Cas9 binding.

Cas9 ribonuclease For SaCas9 3.1 kb in length [15], for SpCas9 4.2 kb [51].

To insert the DNA sequences into an AAV vector, the sequence has to be placed between two 145 bp ITRs. These ITRs are necessary for the integration of the DNA into the genome of an infected cell [79].

Moreover, a fluorescent marker gene (e.g. for a Green fluorescent protein) can be fused to either the guide RNA, or to the Cas9. Since AAV is 4.7 kb, for a single AAV vector with SaCas9 there would be enough space for plasmid insertion [86].

For the double vector system, SpCas9 has to be placed separately with the U6 promoter in one plasmid, and the gRNA (crisprRNA and tracrRNA) together with a fluorescent marker in another.

5.1.2. Confirmation of Knockout

In order to verify that the gene knockout was successful, we plan to examine two levels of gene regulation, the DNA level and the protein level. These experiments will first be performed on an *in vitro* cell culture, and afterwards on the retinal cells of robins post-mortem. Only when both of these experiments give positive results, can the gene knockout be deemed successful. It is also useful to test for the full elimination of the protein when the gene is knocked out, since even when a gene is definitely knocked out, the residual proteins present in the cell might have a long enough lifetime to still be functional [68]. Genetic redundancy could be a factor as well, if more than one gene is responsible for cryptochrome expression, knockout of one of the genes could not be able to stop the expression.

To examine the gene knockout at DNA level, targeted next generation sequencing (NGS) methods are ideal, such as Amplicon sequencing using Illumina technologies (Illumina AmpliSeq), since it has a very high accuracy, shorter time and low DNA input (1 ng to 100 ng). With Illumina, designed or customised panels are available, that include PCR-based library preparation for a relatively easy and fast workflow. For Amplicon sequencing, PCR is used to amplify the targeted sequences with primers and create the fragments, so-called amplicons. Next, these amplicons are partially digested to eliminate the primer ends, so that they will have blunt ends. The blunt ended fragments are ligated to Illumina index adapters and afterwards the samples can be sequenced in an Illumina sequencing system (e.g. MiniSeq) and compared to the reference gene to determine if the knockout has been successful [57].

For determining the protein elimination, Western blot is the most suitable technique. Western blot involves the separation of the proteins by their molecular weight in SDS-Page electrophoresis. Next, the proteins are transferred to a special membrane for immunostaining. To this membrane complementary monoclonal primary and secondary antibodies are administered. If a protein of interest is present, an antibody-protein complex will be formed. Due to the labelling of the antibodies, the complex formation could be visualised, using e.g. fluorescent labels, and the presence of the proteins will be detected [89].

5.1.3. *In Vitro* Tests

In order to do tests with the different plasmids, before injecting it into the eye of a robin, we need to establish a cell culture of the retinal cells of a robin. This has already been done with the retina of rats [56] and humans [50], and we propose to use a similar procedure for the retina of the robin. An explant culture of the retina contains all the different cells that are present in the eye, which makes it suitable for testing the gene therapy, because it is anatomically similar to the eye and easily accessible.

The viral vectors can be administered by injecting it into the fluid, in which the explant is located. The transduction rate can be assessed with the use of optical microscopy. This is done by taking a photograph of the explant through a microscope and counting the cells that express a fluorescent marker [26]. However, this is only possible if a fluorescent marker is transduced in the vector. An alternative is knocking out a certain protein which is present in all the types of cells and using immunostaining, e.g. Western blotting, to determine the proportion of cells expressing the targeted gene [44, 90].

The described methods for knockout confirmation are used to determine how successful the knockout of the cryptochrome proteins was. The vector or vectors for the *in vivo* knockout are selected based on their transduction and knockout efficiency, which depends on the indel mutations that are caused.

5.1.4. Administering the Viral Vector *In Vivo*

Before the viral vector is inserted into the eye, the robin has to be anaesthetised. The viral vector has to be injected into the eye between the photoreceptor layer and the retinal pigment epithelium. For this subretinal injection, a syringe is filled with the viral vector with the desired dose. The vitreous membrane can be detached from the retina, by injecting a salt solution into it. The needle of the syringe with the AAV vector is then injected behind the lens. When the tip of the needle has penetrated the retina, i.e. it is between the photoreceptors and the epithelium, the fluid has to be carefully and slowly inserted into the subretinal cavity [87, 90].

An adeno-associated virus with only the marker will be injected in the same way as the AAV vectors with the sequence for guide RNA in the control group.

Using the marker in the AAV vector, the degree of penetration and knockout can be qualitatively determined. If necessary extra injections can be made. Although the eye is an immunoprivileged organ and thus has a lower risk of an immune response [90], this has to be done diligently taking into account the remaining risk of inflammation that comes with every injection [54, 78],

5.2. Behavioural Study

Six groups of robins, each consisting of 15 individuals, will be used. One group is the control group, where none of the cryptochromes are knocked out. There are four groups,

each with one of the four types of cryptochrome knocked out, and one group with all four types of cryptochrome knocked out.

After the knockout, the robins are first tested for migratory behaviour, using their sun and star compass. Subsequently, they are tested for magnetoreception, using the fact that they show migratory behaviour. This experiment will be referred to as the *magnetoreceptory test* or *principal experiment*. In the following paragraphs the setup of the behavioural experiments will be described.

5.2.1. General Setup

Both the motivational and magnetoreceptory experiments share a common basis which is described here. When conducting the behavioural experiments, each bird is placed in a so-called Emlen funnel, see figure 10. This is a cone shaped funnel with an ink-pad at the bottom. The walls are lined with thermal paper [46]. The top of the funnel is enclosed with a lid to keep the birds in the funnel. When the birds try to migrate, they will leave ink marks on the paper. From these marks the migratory direction can be determined after the experiment. This is done by dividing the paper into sections of equal angles, typically 24 sections of 15 degrees, and comparing each section to a reference key [10].

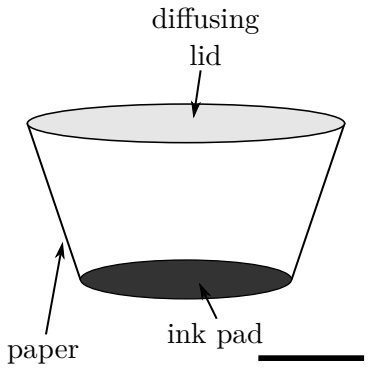

Figure 10: Emlen funnel (20 cm bar for reference)

5.2.2. Motivation for Migration

One of the things that has to be established is that the birds are still motivated to migrate after the *in vivo* gene knockout. This has to be done, because the knockout might have unwarranted side-effects, such as the loss of motivation for migratory behaviour. This might be due to the damage that the needle leaves in the retina [78], or because of the absence of the cryptochrome protein.

This is done using similar methods as Zapka et al. [91]. The first method uses the sun compass. The migratory tendencies of passerines, in particular robins, at dusk, has been

well-documented [1]. The idea is to perform a behavioural study using the Emlen funnel in a field in a rural area. In this case, the Emlen funnels are covered with a transparent plastic net, so that the birds can see the sun. The migratory direction is then determined as described in the previous section.

The second method uses the star compass, which is the ability of birds to navigate based on the stellar configuration. Robins do this by learning the north through the rotational pattern of the night sky [43]. To test this, the Emlen funnels can be placed in a planetarium showing the local night sky, as described in the methods of Zapka et al. [91]. The planetarium consists of a projector and a spherical screen simulating the night sky. To ensure the birds are not using their magnetic sensing ability, electromagnets can be used to distort the magnetic field locally. This magnet has to be placed in the centre of the setup. With this configuration the magnetic field direction differs in each of the Emlen funnels, hence it can be easily observed, if the robins use their magnetic compass instead of the star compass.

5.2.3. Magnetoreceptory Tests

The magnetic sensing of the robins is tested behaviourally as well. Besides the Earth's magnetic field, several artificial fields of geomagnetic strength can be used to establish whether magnetic sensing is used. These experiments have to be conducted in a special facility, which is described in the following paragraphs.

First of all, the Emlen funnels are covered with a mat lid, which diffuses the light and prevents the birds from obtaining any directional clues using their vision [11].

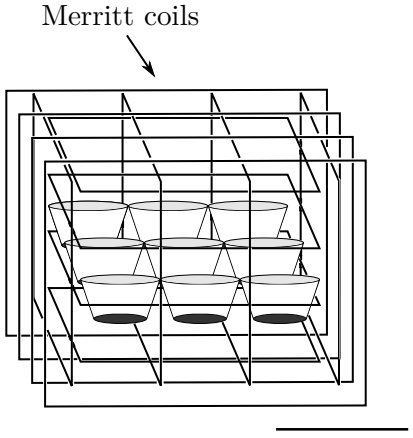

Figure 11: Funnels placed in coil setup (50 cm bar for reference)

In order to control the magnetic field in the experimental setup, the Emlen funnels are placed in an array of magnetic coils, see figure 11. In all three spatial directions, there

are four coils. This constitutes a three-dimensional Merritt four-coil system. With these coils the magnetic field in the funnels can be controlled and kept uniform across the different funnels that are used simultaneously [31]. The coils can be used to establish the different experimental conditions, i.e. different magnetic field directions with geomagnetic strength.

Finally, the complete setup has to be placed in an Faraday cage. It is known that changing electromagnetic fields disrupt the magneto-sensory ability of the European robins [22, 67]. The Faraday cage blocks electromagnetic radiation as well as electric fields, and hence prevents external changing electromagnetic fields from interfering with the magnetoreception of robins [11]. Additionally, as recommended by Hiscock et al. [22], the magnetic field inside the Faraday cage will be recorded during measurements. This is important since the Faraday cage does not block (low frequency) changes in the magnetic field [21].

The experiments with each bird are repeated multiple times. Since the robins belong to the class of nocturnal migratory passerines, tests can only be conducted during night time. At most two experiments can be conducted with each bird on a single day, one after dusk and one before the morning twilight [91].

Every bird will be tested five times, giving a total of 75 measurements per group. A detailed argument for this sample size, based on statistical simulations, is presented in appendix A.

5.2.4. Robin Accommodation

The accommodation for the robins should be close to the natural habitat of robins. This is important for the robins to show natural behaviour during the experiments, and to satisfy ethical regulations. The ethics and the requirements for accommodation are described in more detail in appendix B.

5.3. Result Analysis

In order to draw conclusions from the experimental results, we propose to use the follow statistical tests:

1. Rayleigh z-test

2. Wallraff test

The term *group* is used to refer to the set of measurements of all robins which share the same treatment. For example the measurements of the control group are a group. So are the measurements of all robins where only Cry4 is knocked out. We will call every group which is not the control group, a *knockout group*.

5.3.1. Rayleigh Test

The Rayleigh z-test is used on every group separately. The null hypothesis is that the measurements are drawn from a circular uniform distribution [41, 64]. This can be used to show the existence of a preferred direction, and thus the functioning of a mechanism for orientation. Therefore this test can be used both for the principal experiment, as described in section 5.2.3, to test for functioning magnetoreception, and the sun and star compass experiments, as described in section 5.2.2, to test for motivation to migrate.

The Rayleigh test is only applicable for distributions which are unimodal[4] [41, 64]. Even though the magnetic compass is hypothesised to be an inclination compass rather than a polarity compass [24], the control group measurements of current Emlen funnel experiments [67, 72, 83], give no reason to question the unimodality of measurements for control groups. For the sun and star compass, there is no reason to assume a non-unimodal distribution, and experimental results [1, 43] confirm this. The Rayleigh test performs well for a broad range of unimodal distributions [64], making it a good choice for our experiments.

There is an additional version of the Rayleigh test, which has as the alternative hypothesis that the measurements are sampled from a unimodal distribution around a specific mean direction [64]. This test could be applied to the sun compass test, since Åkesson and Sandberg [1] give the expected migration direction.

5.3.2. Wallraff Test

The Wallraff test is used to compare every knockout group with the control group. The null hypothesis is that the two sets of measurements are sampled from distributions with the same angular dispersion [38]. To be more precise, the angular differences to the mean angle (per group) are calculated. These datasets are then compared using the Kruskal-Wallis rank sum test, which has the null hypothesis that the datasets are drawn from the same distribution [42].

This test will only be used for the principal experiment. There it will be used to show that a knockout group has a differently distributed angular dispersion when compared to the control group, and therefore that the magnetoreception was influenced. Different estimates for the angular dispersion like the length of the mean direction vector, can then be compared to show which group had a larger angular dispersion. This could found that the knockout group has a larger angular dispersion and thus impaired magnetoreception.

R. Wiltschko, Munro, et al. [81] claim that there is a second mechanism for magnetoreception giving a “fixed direction” response. The migratory direction was westerly and not season dependent. The Wallraff test would not be able to distinguish this behaviour from a control group if the accuracy of the second compass is reasonably similar to that of the RPM. If such migration in a different, seasonally incorrect, direction

[4] A unimodal distribution is a distribution which is centred around a single point.

is observed, the Watson U^2 test [41], or the Mardia-Watson-Wheeler [52] test will be used. These tests have as alternative hypothesis that the control and knockout group are drawn from different distributions. Besides angular dispersion, they also take the mean angle into account. Therefore they would be able to distinguish the two mechanisms of magnetoreception. In this case one would be able to conclude that the cryptochrome knockout has *some* effect on the magnetoreception. This is clearly a weaker conclusion, justifying the choice to first use the Wallraff test.

6. Risk Assessment

As usual, we might be confronted with unforeseen setbacks. This section elaborates on the stages in the experiment that might not go as planned and describes appropriate measures.

First of all, whilst administering the viral vector, the retina could get damaged, leading to impaired visual sight. The bird could then not be used for the proposed experiment, since the motivation to migrate needs to be tested using its sun and star compass which in turn require proper visual sight. However, since many studies have used the method of subretinal injection for successful AAV viral vector delivery in the retina, e.g. in mice [3, 54, 90] and in humans [7, 88], we are confident that the method of subretinal injection will not jeopardise proper functioning of the retina.

Another risk is the development of an immune response against the viral vector. Since most research concerning the delivery of a viral vector through subretinal injection focuses on mice and humans, little to no research is done into immune responses to subretinal AAV delivery in European robins, or even birds in general. If such an immune response would occur, another AAV serotype will be used for the CRISPR-Cas9 delivery. Lowering the AAV dose could decrease immune responses [3]. In case all serotypes would cause an immune response, immunosurpressive drugs will be considered.

It is also possible that the transduction of the viral vector is not sufficient, resulting in a non-significant knockout. In this case, a higher dose can be injected or a new vector can be designed. The latter can be done by using a different AAV serotype. Since all serotypes differ in tropism and transduction, see section 4.4.3, the serotype used initially might have been unsuitable for retinal cells of European robins. Another option is to grow retinal cells *in vitro* and do a knockout directly using CRISPR-Cas9, without the AAV. These cells can subsequently be injected in the retina, resulting in a "two-step" knockout instead of the "single-step" *in vivo* knockout.

The off-target effects are another risk of the AAV delivery of the gene modification machinery. Too many off-target DNA-cuts by the nuclease could lead to a decrease in the motivation to migrate or might affect the visual sight of the birds. The amount of off-target effects could be reduced by changing the sgRNA used to guide the nuclease or by using another nuclease and hence a different PAM, see section 4.4.1.

It is also possible that the penetration of the knockout is significant, but not enough to completely disable the magnetic sensing.[5] One solution is to use more AAV injections. This increases the risk of damaging the retina and increases the risk of an inflammation, see section 5.1.4. Another solution uses the fact that a partial knockout, even when not completely disabling the magnetic sensing, would probably impair it. Therefore the angular dispersion would be larger for the knockout group, which could be detected using the Wallraff test, see section 5.3.2. However, this would require a significant amount of extra tests, see appendix A.

As noted in section 5.2.2, another problem is that the robins could lose their motivation to migrate. The two main causes for this could be damage to the retina or the absence of cryptochrome. Furthermore, severe damage to the retina could also cause an inability to use sun and star compass. If the loss of motivation is caused by a lack of cryptochrome, this would be quite an interesting finding by itself, since motivation for migration and the circannual clock are not yet fully understood [33]. This could even be an opportunity to conduct experiments concerning the circadian clock and the circannual clock, but this is outside the scope of this proposal. Thus, in this case, it is important to establish that the lack of motivation for migration or the inability to use all three compasses, is not caused by damage to the retina. First, electroretinography can be used to assess the retinal functioning [4]. Secondly, a behavioural test like one of the tests described in Kelber et al. [29] can be used to show the robins' ability to see colour.

Finally, a disruption in the conditions during the principal experiment could interfere with the robins' ability to orient using the magnetic compass. The experiments are done inside a Faraday cage to block electric fields and electromagnetic radiation from the outside. Furthermore, as recommended by Hiscock et al. [22], we will record the the magnetic field before and during the behavioural experiments, to detect disturbances in the magnetic field.[6]

7. Importance and Impact

The proposed project aims to add a significant contribution to the solution of one of the major open questions of sensory biology [24]. There has been some evidence that the protein cryptochrome is involved in the magnetoreception of European robins through the radical pair mechanism. However, it still remains unclear which type of cryptochrome is responsible. Even though there is a high attraction towards cryptochrome 4, depending on the research group, results vary drastically [20, 53, 55]. As already discussed in the introduction, see section 2, the current evidence for the involvement of cryptochrome in the magnetoreception is mainly circumstantial. The proposed project would really provide

[5] Assuming the cryptochrome is in fact required for this.

[6] This is important because a Faraday cage does not block relatively low frequency changes in the magnetic field [21].

direct experimental evidence for cryptochrome's involvement in the magnetoreception. Moreover, the proposed project would establish, in case cryptochrome is involved, which type of cryptochrome is involved, this answering this long-debated question.

Furthermore, the RPM is seen as one of the cornerstones of quantum biology. However, it has not yet been established beyond reasonable doubt that the proposed radical pair mechanism is indeed involved in the magnetoreception of European robins. Establishing this will be a major breakthrough in the field of quantum biology [40]. Furthermore, establishing that the magnetoreception of robins is indeed a quantum mechanical process, it might give an incentive to explore other complex biological processes from a quantum mechanical perspective. Additionally, due to the high energy fluctuations at room temperature, compared to the energy in quantum mechanical systems, quantum effects are not expected at room temperature. It is still an enigmatic question how the biological systems at high temperatures could harvest these effects, especially at temperatures as high as 40 degrees Celsius in case of *Erithacus rubecula*.

Finally, if successful, the *in vivo* retinal gene knockout with AAV in robins will open the doors for the novel gene editing techniques in new species.

8. Planning and Cost Estimate

The proposed research covers two periods of four years with a one year overlap, see figure 12, and is therefore suited for two PhDs. The first PhD needs to have a background in molecular biology, since he or she will conduct the gene knockout. This includes the plasmid development, the *in vitro* tests and the administering of the viral vector *in vivo*. The second PhD needs a background in ethology, because he or she will conduct the behavioural study which includes the magnetoreceptory tests and the test for the motivation to migrate, see section 5.2. There is one year overlap because when the first group of birds has a type of cryptochrome knocked out, this group can immediately be tested for magnetoreception and motivation to migrate, while the first PhD starts on the knockout for the second group of birds. In the first half of Year 1, the first PhD has time to prepare for the research. The second half of Year 1 will be devoted to the plasmid development. Similarly, in the first half of Year 4, the second PhD has time to prepare for the research. The rest of Year 4 will be filled with the magnetoreceptory and motivation tests. It is important to mention that the behavioural studies can only be conducted during migratory seasons, i.e. the spring migration from February to April and the autumn migration from September to November [2]. The planning also assumes that necessary literature research is being done throughout the whole PhD.

Table 1 gives an indication for the costs of the personnel and materials needed during the research.

It is assumed that a genetics research laboratory is freely available, including PCR equipment, blotting equipment, an ophthalmic surgical microscope, petri dishes and

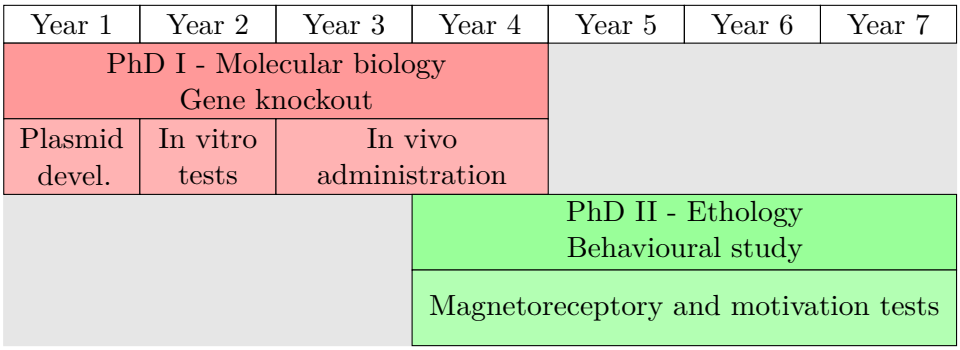

Figure 12: The planning for the proposed research; two subsequent 4-year PhDs with one year overlap.

	Costs (€)
First PhD, 4 years, 1 fte	244 102.00
Second PhD, 4 years, 1 fte	244 102.00
Assistant ornithologist, 5 years, 0.2 fte	86 073.00
Food for robins	45 000.00
Facility for behavioural study	30 000.00
Travel and accomodation costs	10 000.00
Cages for robins	4500.00
Anaesthetics for subretinal injection	4000.00
HEK293 cells for vector production	2400.00
Constructs for AAV CRISPR-Cas9 vectors	1000.00
Total	671 177.00

Table 1: Cost-estimate of the proposed research. The prices are estimated over the period of the entire project.

syringes. The ornithologist guides and assists the second PhD in taking care of the birds. The food and cages for the robins can be bought at Pets Place.[7] The facility for the behavioural study is located in Oldenburg, Germany and has been used for similar experiments. However, we were not able to find out what the costs would be for hiring that facility, hence an estimation is made. The anaesthetics needed for the subretinal injection can be bought via Sigma-Aldrich.[8] These are 1 gram Xylazine for €55.80 and 1 gram Ketamine for €3780. Furthermore, the HEK293 cells for growing the cell cultures can also be bought via Sigma-Aldrich. All AAV and CRISPR-Cas9 constructs can be bought via Addgene.[9] The plasmids are €75 each; we need plasmids for the AAV, Cas9 and for the gRNA (for each type of cryptochrome) together with the markers. The AAV serotype is €380. The first PhD can create the necessary viral vectors from these constructs.

[7] https://petsplace.nl/
[8] https://www.sigmaaldrich.com/nederland.html
[9] https://www.addgene.org/

A. Estimation of Sample Size

We estimated the number of experiments that need to be conducted to reach significance levels of five and one percent, using statistical simulations.

A.1. Assumptions

In order to do this, we assume that the measurements for a group of robins with working magnetoreception, like the control group, is distributed according to the Von Mises distribution. This seems like a reasonable approximation, since the distribution of measurements is expected to be unimodal, see section 5.3.1, and the Von Mises distribution closely approximates the wrapped normal distribution. We do not account for the discretisation of measurements, see section 5.2.3.

Both tests do not depend on (mean) direction, but only on angular dispersion, so the location parameter μ is taken 0. We estimated the concentration parameter κ from the control group measurements of Schwarze et al. [67] and Thalau et al. [73], using the approximation

$$\widehat{\kappa} = \frac{2r - r^3}{1 - r^2} \tag{1}$$

where r is the length of the mean direction, which is specified by both papers. For Schwarze et al. [67] (with $r = 0.43$) this gives $\widehat{\kappa} \approx 0.96$, while for the autumn measurements of Thalau et al. [73] (with $r = 0.91$) this gives $\widehat{\kappa} \approx 6.2$. Other papers like R. Wiltschko, Thalau, et al. [83] find r-values in between. This extremely high variation in angular dispersion between experiments makes it very hard to make a good approximation of κ. For κ, we use 2.

We will write α for the desired significance level, and β for the maximal accepted probability to not reach this significance level.

A.2. Rayleigh Test

First of all, we want to reject the null hypothesis[10] of the Rayleigh test for groups with intact magnetoreception. To find the required sample size n, we wrote a simulation in R using the package `circular`, which is shown in listing 1.

Listing 1: Simulation to determine the minimal sample size for the Rayleigh test, to distinguish samples from the Von Mises distribution with $\kappa = 2$ from uniform.

```
library(circular)
library(glue)

# Terms:
```

[10] The null hypothesis is that the measurements are drawn from a circular uniform distribution, see section 5.3.1.

```
# - sampled data here refers to the measurements on one group of robins
#
# Rayleigh z-test
# H0: the sampled data is uniformly distributed
# H1: the sampled data is not uniformly distributed
#
# Assumptions:
# - In order to use the Rayleigh z-test we have to know that the
#   sampled data is unimodal. There is no reason to expect
#   otherwise based on earlier research.
# - For this simulation we assume that the measurements are
#   sampled from the Von Mises distribution with shape parameter
#   `kappa = 2`
#
# Goal:
# Determine the minimal sample size $n$ such that, given that the
# measurements are indeed sampled from Von Mises(`kappa`), we have
# a chance of `1 - beta` to be able to conclude, using the
# Rayleigh test with significance level `alpha`, that the
# measurements are not sampled from a uniform distribution. (That
# is, robins which do show magnetoreception can be shown to have
# functioning magnetoreception.)

ITER <- 10000
# maximum sample size to try
# complexity of the simulation is linear in this
N_MAX <- 30
# the step size for our sample size `n`
# higher values speed up the simulation, but the results only
# include sample sizes which are multiples of this
N_STEP <- 1
LENGTH <- N_MAX/N_STEP
# significance level
alpha <- 0.01
# max chance that under the made assumptions we aren't able to reject H0
beta <- 0.01
# shape parameter of Von Mises distribution
kappa <- 2

# `count` counts per tested sample size the number of iterations in which H0
# is accepted
count = rep(0, LENGTH)
for (i in seq(1, ITER)) {
    # sample `N_MAX` measurements
    data <- rvonmises(N_MAX, circular(0), kappa)
    # do the test for increasing portions of the just sampled data
    for(j in seq(1, LENGTH)) {
        n <- j*N_STEP
        res <- rayleigh.test(data[1:n])
        p <- res$p.value
```

```
        if (p >= alpha) {
            count[j] <- count[j] + 1
        }
    }
}

avg_counts = count/ITER
save(list = c("ITER", "N_MAX", "N_STEP", "alpha", "beta", "kappa",
    "avg_counts"), file = "Rayleigh_von_Mises_sample_size.Rdata")


print(glue("Average number of zero-hypothesis acceptions: {avg_counts}"))
first_good_n = min(which(avg_counts < beta))*N_STEP
print(glue("First large enough sample size: {first_good_n}"))
```

With $\alpha = \beta = 0.01$ this simulation returns that $n = 20$ is the minimal number of measurements required.

A.3. Wallraff Test

If a knockout completely disables magnetic sensing, so that the measurements for this knockout group are drawn from a circular uniform distribution, we want to conclude, using the Wallraff test that the control group and knockout group have different angular dispersions. This is equivalent to rejecting the null hypothesis of the Wallraff test, see section 5.3.2. To find the required sample size n, a second simulation was used. This is shown in listing 2. Here both 0.05 and 0.01 are tested as the values of α and β.

Listing 2: Simulation to determine the minimal sample size for the Wallraff test, to distinguish samples from the Von Mises distribution with $\kappa = 2$ from samples from the circular uniform distribution.

```
library(circular)
library(glue)

# Terms:
# - *control* are the measurements from the control group
# - *alternative* are the measurements from the group we want to
#   test (robins with knockout)
#
# Wallraff test
# H0: control and alternative are sampled from distributions with
#     identical angular dispersion
# H1: control and alternative are sampled from distributions with
#     different angular dispersion
#
# Assumptions:
# - For this simulation we assume that control is sampled from the
#   Von Mises distribution with shape parameter `kappa = 2`
# - For this simulation we assume that alternative is sampled from
```

```
#   the uniform distribution
#
# Goal:
# Determine the minimal sample size $n$ such that, given that the
# assumptions are satisfied, we have a chance of `1 - beta` to be
# able to conclude, using the Wallraff test with significance
# level `alpha`, that control and alternative are sampled from
# distributions with different angular dispersion. (That is, show
# that the knockout robins have larger angular dispersion/show
# less magnetoreception.)

ITER <- 10000
# maximum sample size to try
# complexity of the simulation is linear in this
N_MAX <- 100
# the step size for our sample size `n`
# higher values speed up the simulation, but the results only include
# sample sizes which are multiples of this
N_STEP <- 5
LENGTH <- N_MAX/N_STEP
# significance levels (we test multiple in one go since the simulation is
    ↪ quite
# slow and we don't want to run multiple times with different parameters)
alpha <- c(0.05, 0.01)
# max chances that under the made assumptions we aren't able to reject H0
beta <- c(0.05, 0.01)
# shape parameter of Von Mises distribution for control
kappa <- 2

# `count` counts per tested sample size the number of iterations in which H0
# is accepted
count = matrix(0, nrow=2, ncol=LENGTH)
for (i in 1:ITER) {
    # sample `N_MAX` measurements; `data1` is control, `data2` alternative
    data1 <- rvonmises(N_MAX, circular(0), kappa)
    data2 <- rcircularuniform(N_MAX)
    # do the test for increasing portions of the just sampled data
    for(j in 1:LENGTH) {
        n <- j*N_STEP
        data_slice = list(data1[1:n], data2[1:n])
        res <- wallraff.test(data_slice)
        p <- res$p.value
        for (k in seq_along(alpha)) {
            if (p >= alpha[k]) {
                count[k, j] <- count[k, j] + 1
            }
        }
    }
}
```

```
avg_counts = count/ITER
save(list = c("ITER", "N_MAX", "N_STEP", "alpha", "beta", "kappa", "avg_
    ↪ counts"), file = "Wallraff_uniform_vs_von_Mises_sample_size.Rdata")

for (k in seq_along(alpha)) {
    print(glue("alpha: {alpha[k]}"))
    print(glue("Average number of zero-hypothesis acceptions: {avg_counts[k
        ↪ ,]}"))
    for (b in beta) {
        cat("\n")
        print(glue("alpha: {alpha[k]}"))
        print(glue("beta: {b}"))
        first_good_n = min(which(avg_counts[k,] < b))*N_STEP
        print(glue("First large enough sample size: {first_good_n}"))
    }
    cat("\n")
}
```

With $\alpha = \beta = 0.01$ this simulation returns that $n = 55$ is the minimal multiple of five measurements required. Both $\alpha = 0.01, \beta = 0.05$ and $\alpha = 0.05, \beta = 0.01$ give $n = 45$.

As mentioned in section 6, it is possible that only a partial knockout can be achieved. This might not lead to full absence of magnetic sensing, but only heavily impaired magnetic sensing. In this last simulation we estimate the number of tests needed to still reject the null hypothesis of the Wallraff test, if the knockout group's measurements are also drawn from the Von Mises distribution, but with κ a quarter of the κ for the control group. The simulation, which only differs from the previous one in the sampling of `data2`, is shown in listing 3.

Listing 3: Simulation to determine the minimal sample size for the Wallraff test, to distinguish samples from the Von Mises distribution with $\kappa = 2$ from samples with $\kappa = 0.5$.

```
library(circular)
library(glue)

# Terms:
# - *control* are the measurements from the control group
# - *alternative* are the measurements from the group we want to
#   test (robins with knockout)
#
# Wallraff test
# H0: control and alternative are sampled from distributions with
#     identical angular dispersion
# H1: control and alternative are sampled from distributions with
#     different angular dispersion
#
# Assumptions:
# - For this simulation we assume that control is sampled from the
#   Von Mises distribution with shape parameter `kappa = 2`
# - For this simulation we assume that alternative is sampled from
```

```
#   the Von Mises distribution with shape parameter `kappa = 0.5`
#
# Goal:
# Determine the minimal sample size $n$ such that, given that the
# assumptions are satisfied, we have a chance of `1 - beta` to be
# able to conclude, using the Wallraff test with significance
# level `alpha`, that control and alternative are sampled from
# distributions with different angular dispersion. (That is, show
# that the knockout robins have larger angular dispersion/show
# less magnetoreception.)

ITER <- 10000
# maximum sample size to try
# complexity of the simulation is linear in this
N_MAX <- 100
# the step size for our sample size `n`
# higher values speed up the simulation, but the results only
# include sample sizes which are multiples of this
N_STEP <- 5
LENGTH <- N_MAX/N_STEP
# significance levels (we test multiple in one go since the
# simulation is quite slow and we don't want to run multiple times
# with different parameters)
alpha <- c(0.05, 0.01)
# max chances that under the made assumptions we aren't able to reject H0
beta <- c(0.05, 0.01)
# shape parameter of Von Mises distribution for control
kappa <- 2
# shape parameter of Von Mises distribution for alternative
kappa_prime <- kappa/4

count = matrix(0, nrow=2, ncol=LENGTH)
for (i in 1:ITER) {
    # sample `N_MAX` measurements; `data1` is control, `data2` alternative
    data1 <- rvonmises(N_MAX, circular(0), kappa)
    data2 <- rvonmises(N_MAX, circular(0), kappa_prime)
    # do the test for increasing portions of the just sampled data
    for(j in 1:LENGTH) {
        n <- j*N_STEP
        data_slice = list(data1[1:n], data2[1:n])
        res <- wallraff.test(data_slice)
        p <- res$p.value
        for (k in seq_along(alpha)) {
            if (p >= alpha[k]) {
                count[k, j] <- count[k, j] + 1
            }
        }
    }
}
```

```
avg_counts = count/ITER
save(list = c("ITER", "N_MAX", "N_STEP", "alpha", "beta", "kappa", "avg_
    ↪ counts"), file = "Wallraff_von_Mises_vs_quarter_kappa_sample_size.Rdata
    ↪ ")

for (k in seq_along(alpha)) {
    print(glue("alpha: {alpha[k]}"))
    print(glue("Average number of zero-hypothesis acceptions: {avg_counts[k
        ↪ ,]}"))
    for (b in beta) {
        cat("\n")
        print(glue("alpha: {alpha[k]}"))
        print(glue("beta: {b}"))
        first_good_n = min(which(avg_counts[k,] < b))*N_STEP
        print(glue("First large enough sample size: {first_good_n}"))
    }
    cat("\n")
}
```

With $\alpha = \beta = 0.05$ this simulation returns that $n = 55$ is the minimal multiple of five measurements required. Both $\alpha = 0.01, \beta = 0.05$ and $\alpha = 0.05, \beta = 0.01$ give $n = 70$ and $\alpha = \beta = 0.01$ gives $n = 95$.

A.4. Conclusion

Based on these simulations we conclude that 15 birds per group, each tested 5 times, is a reasonable sample size for the principal experiment. This would bring a total of 75 measurements per group, aiming at $\alpha = \beta = 0.01$ for the Rayleigh test and Wallraff test given complete knockout, and at $\alpha = \beta = 0.05$ for the Wallraff test given partial knockout.

The safety margin of $75 - 55 = 20$ measurements is to account for the following errors, ordered with decreasing expected impact:

1. The measurements will be discretised. This will have an averse effect on the performance of the statistical tests.

2. The actual κ might be slightly lower than 2, especially considering the results of Schwarze et al. [67].

3. The measurements might not be distributed along the von Mises distribution.

4. The results here are based on simulations. They might be slightly off by pure chance or because of the imperfection of the random number generator.

B. Ethics and Robin Accommodation

We are highly aware how strict the regulations of the dutch animal experiment committees Dierenexperimentencommissie (DEC) and the Centrale Commissie Dierproeven (CCD) are regarding animal testing. When designing the experiments we have ensured that they comply with the 1986 Treaty 123 titled *European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes* [9].

We give our assurance that in case we receive the grant, all the conditions regarding the robin accommodation and experiments will be kept exactly as the committees expect from us.

For the behavioural experiments we intend to hire the research facility from Animal Navigation group lead by Prof. Henrik Mouritsen at the *Carl von Ossietzky Universität Oldenburg* located in Oldenburg, Germany [27]. Prof. Mouritsen's group has been involved in behavioural experiments on migratory birds with similar setup as ours (e.g. Emlen funnels), therefore the equipment will be readily provided.

Birds will be wild-caught from areas in the close proximity of Oldenburg a few weeks before the experiments start, in order for the robins to get used to living in captivity. The experiments will be performed in migratory seasons. The birds will have a small identification ring on their legs with a number, that will not hinder their movement or flying in any way.

B.1. Accommodation

Birds will be kept in indoor wire cages where the artificial light will be arranged to match the local daily light cycles.

Floor drainage will be provided not to give rise to humidity or mold in the space. Pecking blocks and straw will be provided to the birds.

Since robins are used to living in temperate climate conditions, keeping the temperature in the cages the same as it is outside will be sufficient for the birds.

B.2. Feeding

Robins will be provided suitable fresh food (earthworms, terrestrial invertebrates, berries and seeds) and water at all times in containers at special feeding stations. The containers and food holders will be made in way that will cause no harm to the birds. They will be cleaned and disinfected on a regular basis. The diet will be kept varied based on the season to provide the birds with their nutritional requirements.

Mixed grit will be available as a source of Calcium.

B.3. Euthanasia

After the experiments are conducted, the birds will be euthanised with hypoxia produced by inert gases, nitrogen (N_2) and argon (Ar). This method appears to kill all birds in less than 3 minutes and none of the animals showed signs of distress compared to birds that were exposed to CO_2 [34]. It would be probably one of the most preferable methods to be used on bird species like robins.

C. Quantum Mechanical Simulation

This appendix treats the method by which the quantum mechanical simulations were performed. As has been described in the background, see section 4.2, the system consists of two electrons and two nuclei and our model involves the Zeeman interaction and the hyperfine interaction. We assume both the electrons and the nuclei are spin $\frac{1}{2}$. Therefore the state space is $\mathbb{C}^2 \otimes \mathbb{C}^2 \otimes \mathbb{C}^2 \otimes \mathbb{C}^2 = \mathbb{C}^{16}$. Let us write $\hat{\mathbf{S}}_1$ and $\hat{\mathbf{S}}_2$ for the spins of the first and second electron, respectively. Let us also write $\hat{\mathbf{I}}_1$ and $\hat{\mathbf{I}}_2$ for the nuclear spins of nuclei. The Hamiltonian we simulated was

$$\hat{H} = \omega_1 \mathbf{B} \cdot \hat{\mathbf{S}}_1 + \omega_2 \mathbf{B} \cdot \hat{\mathbf{S}}_2 + \hat{\mathbf{S}}_1 \cdot A\hat{\mathbf{I}}_1 + \hat{\mathbf{S}}_2 \cdot C\hat{\mathbf{I}}_2, \tag{2}$$

where $\mathbf{B}$ is the magnetic field vector, ω_1 and ω_2 are parameters describing the strength of the Zeeman effect and A and C are matrices encoding the strength and anisotropy of the hyperfine interaction. The Hamiltonian was written in matrix form and implemented in python using numpy.

Because we have uncertainty in the initial conditions we used density matrices. The time evolution is the described by the von Neumann equation

$$i\hbar \frac{\partial \rho}{\partial t} = [\hat{H}, \rho],$$

where ρ is the density matrix. For time independent systems, such the one we use, the solution to this equation is

$$\rho(t) = e^{-\frac{i\hat{H}t}{\hbar}} \rho(0) e^{\frac{i\hat{H}t}{\hbar}}.$$

This function was implemented in python using the complex exponential function from numpy.

Because we are only interested in the spin component of this system, we took all nuclear spin states as equally probable in our initial conditions. However, the spin component was in the singlet state. The parameters we used were $\omega_1 = 1$, $\omega_2 = 1$, $|\mathbf{B}| = 5 \times 10^{-5}\,\mathrm{T}$ (the Earth's magnetic field strength) and for the hyperfine interactions

$$A = \begin{pmatrix} 5 \times 10^{-4}\,\mathrm{T} & 0 & 0 \\ 0 & 5 \times 10^{-4}\,\mathrm{T} & 0 \\ 0 & 0 & 5 \times 10^{-4}\,\mathrm{T} \end{pmatrix},$$

and

$$B = \begin{pmatrix} 10 \times 10^{-4}\,\mathrm{T} & 0 & 0 \\ 0 & 10 \times 10^{-4}\,\mathrm{T} & 0 \\ 0 & 0 & 0 \end{pmatrix}.$$

The angles between the magnetic field and the z-axis were 0° and 90° respectively for the two simulations.

The simulations were run over the course of 2.5 µs with 10000 timesteps.

List of Abbreviations

AAV adeno-associated virus 5, 15–20, 25–27, 29

CCD Centrale Commissie Dierproeven 37

DEC Dierenexperimentencommissie 37

gRNA guide RNA 18, 19, 29

HDR homology directed repair 16

ITR inverted terminal repeat 16, 17, 19

NGS next generation sequencing 19

NHEJ nonhomogolous end joining 16

PAM protospacer adjacent motif 15–17, 25

RPM radical pair mechanism 6, 7, 9, 24, 26, 27

SaCas9 *Staphylococcus aureus* Cas9 17–19

sgRNA single guide RNA 15, 17, 25

SpCas9 *Streptococcus pyogenes* Cas9 17–19

ssDNA single stranded DNA 16

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