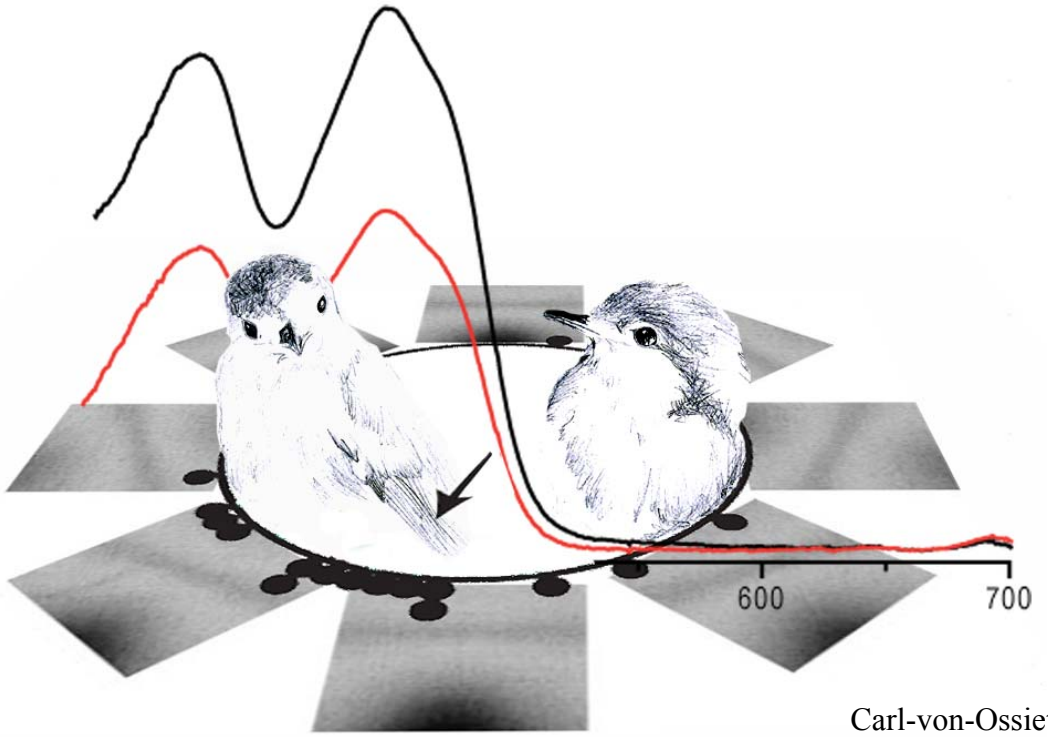
Zur Homepage der Dissertation

Magnetic orientation in migratory birds

– the question of perception and neuronal integration



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Ph.D. thesis

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Magnetic orientation in migratory birds

– linking behavioural phenomena with underlying molecular and neuronal mechanisms

Von der Fakultät für Mathematik und Naturwissenschaften der Carl von Ossietzky Universität Oldenburg zur Erlangung des Grades und Titels eines Doktors (Dr. rer. nat.) angenommene Dissertation

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Summary of the Ph.D. thesis

Migratory birds are superb navigators. The accuracy by which migratory birds return to the same breeding site year after year on very similar dates is remarkable. To perform this impressive task, birds draw on a range of cues that help them find their way; namely celestial cues such as the sun and the stars, and the Earth's magnetic field. During my Ph.D. project, I focused on magnetic compass orientation in night-migratory birds. In particular, I concentrated on the question: How can migratory birds perceive directional information from the Earth's magnetic field, and how do they process and integrate this information so that they can use it for compass orientation during their migratory journeys?

I used an integrative approach to investigate light-dependent magnetic compass orientation in birds. My investigations covered the entire process of magnetic compass sensing starting with the physical properties of the most likely primary receptor molecule going all the way to a potential higher integration centre for magnetic compass information in the brain. To do this, I used a wide range of behavioural, neurobiological and molecular methods.

Most of my studies start out with careful observations of the birds' orientation behaviour under controlled conditions. I used our newly designed experimental setup that allows for real time monitoring and analysis of the bird's behaviour and orientation performance under controllable magnetic field conditions. In this setup, garden warblers (*Sylvia borin*) tested in autumn under the natural magnetic field showed clear orientation towards their natural south-westerly migratory direction, whereas birds tested in a zero-magnetic field oriented randomly. So how did the birds under natural magnetic conditions perceive the reference direction provided by the Earth's magnetic field?

While carefully analysing the behaviour of both experimental groups we identified a very specific "head scanning" behaviour in addition to the previously described and well characterised migratory-restlessness behaviour. To test, if head scanning is linked to magnetosensing, we compared the head scanning frequencies of birds, tested in a completely compensated zero magnetic field with control birds experiencing the natural geomagnetic field. We found an almost threefold increase in head-scanning frequency for birds tested in the completely compensated zero magnetic field. Furthermore, we noticed a significant increase in head-scanning frequency shortly before initiating their first period of migratory restlessness behaviour on each given night, suggesting that migratory birds carefully determined the reference direction of the ambient magnetic field just before starting their orientation

behaviour. Furthermore, head scanning makes the bird correct its orientation in the NMF. Based on these findings, we suggest that head-scanning behaviour is directly involved in the process of sensing the reference direction of the Earth's magnetic needed for magnetic compass orientation. This finding shows that their magnetic compass sensor is located in the head and it fits well with behavioural evidence and theoretical considerations suggesting that radical-pair processes in differently oriented, light-sensitive molecules of the retina could enable migratory birds to perceive the magnetic field as a visual pattern. In this context, head-scanning suggests that movement of the magnetically modulated pattern on the retina is facilitating its detection by the birds.

The cryptochrome protein family has been suggested as the most likely candidate class of primary magnetic compass receptors, because they are the only class of molecules known in vertebrates that are thought to fulfil the necessary biophysical characteristics. Therefore the first question we investigated was whether cryptochromes exist in the retina of migratory birds. During my Ph.D. studies, I identified, cloned and sequenced four different members of the cryptochrome family in the retina of migratory garden warblers: Cry1a, Cry1b, Cry2 and Cry4. Mapping the retinal expression pattern of garden warbler Cry1a by means of immunohistochemistry showed that Cry1a is predominantly expressed in the cytosol of ganglion cells, displaced ganglion cells and in the photoreceptor layer. Furthermore, we found striking differences in cryptochrome expression levels between migratory and non-migratory birds at night. While Cry1a expression levels in night-migratory birds were most prominent during the night, Cry1a expression levels in non-migratory control species decreased to background at night. We also mapped the neuronal activity pattern of retinal cells during night-time magnetic orientation and found perfect colocalisation of activity marker genes and Cry1a. Thus, our study provided the first experimental demonstration of the presence of a putative light-mediated primary magnetoreceptor molecule in the eye of migratory birds. But do migratory bird cryptochromes really fulfil the fundamental biophysical characteristics assumed for the primary receptor molecule in the model calculations on which the light-mediated magnetic compass hypothesis is based? To test whether or not these crucial assumptions can be experimentally validated, it is necessary to study the protein in isolation. Therefore, I recombinantly expressed cryptochromes of garden warblers in large amounts. This was the first successful expression of an animal cryptochrome in a heterologous cell-system with its flavin cofactor built in (holoprotein). Having the cryptochrome holoprotein of a migratory bird at hand, we were able to show that light-excitation leads to radical pair formation and that these radicals are very long lived (~ 10 milliseconds) and therefore very well suited for detecting the

direction of Earth-strength magnetic fields. If cryptochromes in the eyes of migratory birds are primary magnetoreceptors, then a light-dependent signal will be sent to the brain. So, the next question we asked was which brain area(s) may be involved in processing information relevant for magnetic compass orientation?

Behavioural molecular mapping is based on visualising neuronal activation patterns in the brain, thus allowing to identify brain areas that are active during a specific behavioural task (in our case: magnetic compass orientation). We used this method to identify a distinct brain area in the anterior forebrain of night-migratory birds, which showed high levels of neuronal activation at night time during magnetic orientation but not during the day. We termed this area "Cluster N" ("N" for night). In non-migratory zebra finches (*Taeniopygia guttata*) and canaries (*Serinus canaria*), Cluster N did not show enhanced levels of neuronal activation, neither during the day nor during the night. Cluster N is located either adjacent to or as part of the visual Wulst, an area known to integrate visual input. Thus, we suspected a functional relation of Cluster N to visual processing. To investigate this assumption, we tested European robins (*Erithacus rubecula*) wearing light tight eye covers to prevent any visual stimulus from reaching the eye. This resulted in a significant decrease to almost baseline level of neuronal activation in Cluster N. From these findings we conclude that Cluster N is a brain area active specifically during night-vision in night-migratory birds. This finding is especially interesting in the context of light-dependent magnetoreception: night-migratory birds may require a specialised forebrain area, such as cluster N, for enhanced night-vision and/or visual night-time navigation. The navigational signals sensed and the information processed could be used for star compass and/or magnetic compass orientation. To further investigate these hypotheses, we examined the effects of lateralisation, red light and various magnetic field conditions which are known to affect orientation performance on neuronal activation patterns of Cluster N. Our data indicate a significant dominance of Cluster N activation in the right hemisphere of European robins. Garden warblers tested under different magnetic field conditions and under monochromatic red light did not show significant differences in cluster N activation. These results do not allow us to conclude whether or not Cluster N is really involved in processing magnetic compass orientation.

My thesis provides evidence on the behavioural, molecular as well as cognitive level supporting a light-mediated magnetoperception mechanism in night-migratory birds. All findings I obtained during my Ph.D. studies are in line with the hypothesis that magnetically modulated intensity patterns may be perceived by the visual system. In other words, night-migratory birds may literally "see" the direction of the Earth's magnetic field.

Zusammenfassung der Dissertation

Zugvögel sind ausgezeichnete Navigationskünstler. Die Genauigkeit, mit der Zugvögel Jahr für Jahr an genau denselben Brutplatz zurückkehren, ist bemerkenswert. Im Zuge ihrer außergewöhnlich präzisen Kompassorientierung stützen sie sich auf die Himmelskörper Sonne und Mond sowie auf das Erdmagnetfeld. Im Rahmen meiner Doktorarbeit habe ich mich speziell mit der Magnetkompassorientierung von nachtziehenden Zugvögeln beschäftigt. Insbesondere habe ich mich mit folgender Frage auseinandergesetzt: Wie können Zugvögel die Richtungsinformation des Erdmagnetfeldes wahrnehmen und wie wird diese Information im Gehirn der Vögel verarbeitet, um sie zur Kompassorientierung nutzen zu können?

In einem integrativen Ansatz habe ich die lichtabhängige Magnetkompassorientierung bei Zugvögeln näher untersucht. Meine Untersuchungen umfassen den gesamten Prozess der Magnetkompassorientierung, beginnend mit den physikalischen Eigenschaften der vermeintlichen Rezeptormoleküle bis hin zur Identifizierung potentieller Verarbeitungszentren von Magnetkompassinformationen im Gehirn. Um dieser komplexen Fragestellung nachgehen zu können, habe ich eine breitgefächerte Kombination aus molekular-, verhaltens- und neurobiologischen Methoden gewählt.

Die Grundlage meiner Experimente bildete eine genaue Beobachtung des Orientierungsverhaltens der Vögel. Hierzu nutzte ich unseren neu entwickelten Versuchsaufbau, der eine detaillierte Beobachtung und die gleichzeitige Auswertung der Orientierungsleistung von Vögeln unter kontrollierten Magnetfeldbedingungen in Echtzeit ermöglicht. In dieser Versuchsvorrichtung orientierten sich Gartengrasmücken (*Sylvia borin*) während des Herbstzugs ihrer natürlichen Zugrichtung entsprechend nach Süd-West. Im Gegensatz dazu zeigten Gartengrasmücken, die in einem komplett kompensierten Null-Magnefeld getestet wurden, keine klare Richtungspräferenz. Wie aber nahmen die Vögel, die unter natürlichen Magnetfeldbedingungen getestet wurden, die Referenzrichtung des Erdmagnetfeldes wahr? Bei der genauen Analyse der Verhaltensdaten beider Versuchsgruppen fiel uns neben der bekannten *Zugunruhe* eine sehr charakteristische Scan-Bewegung des Kopfes auf. Um zu analysieren, ob diese Scan-Bewegung mit der Magnetfeldwahrnehmung zusammenhängt, haben wir die Kopf-Scan-Frequenz der Vögel im kompensierten Null-Magnetfeld mit der von Vögeln im natürlichen Magnetfeld verglichen. Wir stellten einen nahezu dreifachen Anstieg der Scan-Frequenz bei Vögeln fest, die im kompensierten Null-Magnetfeld getestet wurden. Darüber hinaus beobachteten wir eine signifikante Zunahme der Scan-Frequenz kurz vor Beginn des ersten *Zugunruhe*-Intervalls jeder Versuchsnacht, was

vermuten lässt, dass Vögel die Referenzrichtung des umgebenden Magnetfeldes kurz vor Beginn ihres Orientierungsverhaltens sehr sorgfältig ermitteln. Wir sind der Ansicht, dass die Scan-Bewegung des Kopfes im funktionellen Zusammenhang mit der Wahrnehmung der für die Magnetkompassorientierung notwendigen Referenzrichtung des Erdmagnetfeldes steht. Unsere Daten zeigen, dass der Sensor zur Magnetkompassorientierung im Kopf lokalisiert ist. Dies stimmt sowohl mit weiteren Verhaltensdaten als auch mit theoretischen Überlegungen überein, die postulieren, dass Radikalpaar-Prozesse in unterschiedlich orientierten lichtsensitiven Molekülen der Retina (Netzhaut) dem Vogel die Wahrnehmung des Erdmagnetfeldes als visuelles Muster ermöglichen. Das beobachtete Scan-Verhalten könnte dazu dienen, das durch das Magnetfeld modulierte Muster (und damit die Referenzrichtung) wahrzunehmen.

Proteine aus der Familie der Cryptochrome (Cry) wurden als potentielle Rezeptorkandidaten vorgeschlagen. Demnach lautet die erste Frage, die wir uns in diesem Zusammenhang stellten: Sind Cryptochrome in der Retina von Zugvögeln vorhanden? Während meiner Promotion konnte ich vier verschiedene Cryptochrome in der Retina von Gartengrasmücken nachweisen: Cry1a, Cry1b, Cry2 und Cry4. Eine immunohistochemische Untersuchung des Expressionsmusters von Cry1a in der Retina zeigte, dass diese Moleküle überwiegend im Cytoplasma von Ganglienzellen, dislozierten Ganglienzellen sowie Photorezeptoren exprimiert werden. Darüber hinaus fanden wir auffällige Unterschiede in der Expressionsstärke von Cry1a beim Vergleich von Zugvögeln und Standvögeln. Während die Expression von Cry1a in nachtziehenden Gartengrasmücken während der Nacht am höchsten war, sank die Expression bei Standvögeln nachts auf das Basisniveau ab. Wir untersuchten zudem das neuronale Aktivitätsmuster von Retinazellen während der nächtlichen Magnetkompassorientierung, wobei eine perfekte Colokalisierung von Aktivitätsmarkern und Cryptochrom zu erkennen war. Unsere Studie liefert den ersten experimentellen Nachweis eines möglichen lichtabhängigen Magnetfeldsensors im Auge eines Zugvogels. Weist jedoch das Zugvogel-Cryptochrom auch die biophysikalischen Eigenschaften auf, welche die Hypothese zur lichtabhängigen Magnetkompassorientierung postuliert? Um dies experimentell zu überprüfen, muss das Protein *in vitro* untersucht werden. Dafür habe ich Gartengrasmücken-Cryptochrom im großen Maßstab in einem rekombinanten Zellsystem exprimiert. Dies war die erste erfolgreiche Exprimierung eines Vertebraten-Cryptochroms als Holoprotein (mit gebundenem Cofaktor). Wir konnten zeigen, dass das Zugvogel-Cryptochrom nach einem Lichtreiz langlebige Radikalpaare (Halbwertzeit ~10 Millisekunden) bildet – die entscheidende Voraussetzung dafür, dass diese Moleküle der Wahrnehmung von Richtungsinformation des Erdmagnetfeldes

dienen können. Wenn Cryptochrome im Auge von Zugvögeln als Magnetosensoren fungieren, so müssten Magnetfeldinformationen als lichtabhängiges Signal zum Hirn gesendet werden. Die nächste Frage lautet somit: Welche(s) Hirnareal(e) ist/sind an der Verarbeitung von Informationen, die für den Magnetkompass relevant ist, beteiligt? Die Methode *behavioural molecular mapping* basiert auf der Visualisierung neuronaler Aktivitätsmuster im Hirn und bietet so die Möglichkeit Hirnareale zu identifizieren, die während eines spezifischen Verhaltens (in unserem Fall Magnetkompassorientierung) aktiv sind. Wir konnten im Vorderhirn von Nachtziehern ein Areal identifizieren, das nachts – nicht aber am Tag – ausgeprägte neuronale Aktivität aufweist, das so genannte „Cluster N" („N" wie Nacht). In Zebrafinken (*Taeniopygia guttata*) und Kanarienvögeln (*Serinus canaria*) – beides nichtziehende Vogelarten – zeigte Cluster N weder nachts noch tagsüber erhöhte neuronale Aktivität. Weil Cluster N neuroanatomisch betrachtet entweder ein Teil des visuellen Wulstes oder ein eng angrenzendes Hirnareal ist, vermuteten wir eine funktionelle Verbindung von Cluster N und visueller Verarbeitung. Wir testeten Rotkehlchen (*Erithacus rubecula*) mit lichtundurchlässigen Augenklappen, um zu verhindern, dass ein visueller Stimulus das Auge erreichte. Dies resultierte in einem signifikanten Rückgang neuronaler Aktivität in Cluster N. Wir schlussfolgern daraus, dass Cluster N ein Hirnareal ist, das speziell während des Nachtsehens in Nachtziehern aktiv ist. Dieses Ergebnis ist vor allem im Zusammenhang mit lichtabhängiger Magnetkompassorientierung bedeutsam – möglicherweise benötigen Nachtzügler zum verbesserten Nachtsehen eine Vorderhirnstruktur wie Cluster N. Die so wahrgenommenen Navigationssignale könnten für den Sternen- und/oder Magnetkompass genutzt werden. Wir haben daher die Auswirkungen von Lateralisierung, Rotlicht sowie unterschiedlichen Magnetfeldbedingungen, die bewiesenermaßen die Orientierungsleistung von Zugvögeln beeinflussen, auf das neuronale Aktivitätsmuster von Cluster N hin analysiert. Unsere Ergebnisse zeigen für Rotkehlchen eine eindeutige Dominanz von Cluster N in der rechten Hirnhälfte. Unter verschiedenen Magnetfeldbedingungen sowie unterschiedlichen Lichtbedingungen getestete Gartengrasmücken zeigten keine signifikante Veränderung in der Aktivität von Cluster N. Diese Ergebnisse lassen keinen eindeutigen Schluss darüber zu, ob Cluster N an der Verarbeitung Magnetkompass-relevanter Informationen beteiligt ist.

Meine Dissertation liefert Hinweise auf molekularer, kognitiver sowie Verhaltensebene, die einen lichtabhängigen Mechanismus der Magnetrezeption in Zugvögeln unterstützen. Alle Ergebnisse meiner Arbeit sind im Einklang mit der Hypothese, dass durch das Magnetfeld modulierte Intensitätsmuster vom visuellen System wahrgenommen werden. Mit anderen Worten: Zugvögel können das Magnetfeld möglicherweise buchstäblich „sehen".

Structure of the thesis

My Ph.D. thesis starts with an introductory chapter, summarising our current knowledge about spatiotemporal strategies and orientation cues used by migratory birds and presents my own contributions achieved during my Ph.D. studies in the context of the research field of animal navigation. This is followed by six manuscripts (three of them published, one submitted, one under review and one close to submission) presenting the results of my Ph.D. studies. In addition, the thesis comprises *Supplementary material A & B*, summarising the methodology of the cryprochrome cloning and expression study I carried out within the time of my Ph.D. project.

Focus and aim of the Ph.D. project

During my Ph.D. studies I applied behavioural observation, molecular, biochemical and biophysical analysis, as well as neuroanatomical and neurophysiological concepts to allow insight into certain aspects of the complex phenomenon of magnetic orientation in migratory birds. I concentrated on the following aspects:

(1) *Careful control and analysis of the* ***behaviour*** *during magnetic orientation*. Are there characteristic components of the behavioural repertoire during magnetic orientation, which might indicate a certain magnetoperception mechanism?

(2) ***Molecular characterisation*** *of the putative primary receptor molecule.* Does the putative receptor molecule exist in the retina of migratory birds? Given the identification, can the assumptions, included in the model of light-mediated magnetoperception, be experimentally validated?

(3) *The primary sensory input must be* ***processed and integrated in the brain*** *to successfully perform the* ***cognitive task*** *of magnetic orientation.* What brain areas are involved in integrating and processing information for orientation and navigation?

We focused on two night-migratory bird species in our experiments, namely garden warblers (*Sylvia borin*) and European robins (*Erithacus rubecula*). Non-migratory control species were zebra finches (*Taeniopygia guttata*), canaries (*Serinus canaria*) and Sardinian warblers (*Sylvia melanocephala*). Each of the three specific aspects, I concentrated on during my Ph.D. studies, is expanded on an independent array of specific analytical procedures; however, all three individual approaches are embedded in a coherent overall experimental paradigm and therefore

the results are directly linked to each other and allow for direct comparison on multiple scales, namely: perception (receptor level) – integration and processing (neurophysiologic level) - behaviour ("output" level).

Overall study design and integration of different approaches

The general study design was drafted to meet all prerequisites required by each of the following-up analytical approaches. Especially the method of *behavioural molecular mapping*, applied for analysis of activity dependent gene patterns in the brain during magnetic orientation, demands a carefully controlled behavioural scenario. Any unspecific stimulus or behaviour may be reflected in the activity pattern of the brain and will therefore hamper a fundamental analysis of neuronal activity patterns due to magnetic orientation. A carefully controlled scenario is equally important for neuronal activity analysis in retinal cells expressing cryptochrome, a potential magnetosensitive molecule.

Thus, for all species and questions, the experiments rely on close observation of the bird, especially during the last 60 minutes where consistent performance of the target behaviour (e.g. migratory restlessness or sitting still) is most important. This was followed by dissection of the bird's brain and eye-cups and sectioning of the tissue on a cryostat. Radioactive ***in situ*** **hybridisation** was used to visualise the neuronal activity of the brain, **molecular analyses** and **immunohistochemical staining** were applied to the retinal tissue samples of the same bird. This paradigm enabled me to analyse various aspects of the complex behavioural task of magnetosensing and magnetic orientation on different scales and allowed for final integration and complementation of results at the same time on the same birds (Paper I – VI). This approach also ensured that the number of wild caught birds needed for the experiment was kept as low as possible.

I carried out the experiments addressing the question of **neuronal perception and integration** (Paper IV, V, VI) based on the method of *behavioural molecular mapping* together with Gesa Feenders with distributed responsibilities: For experiments with European robins (*Erithacus rubecula*) and zebra finch (*Taeniopygia guttata*) control birds, I was the primarily responsible person. In these experiments, I applied the entire procedure, from behavioural observation to quantification and analysis of the permanent sections. Experiments on garden warbler (*Sylvia borin*) and additional control species were carried out together with G. Feenders, who was the primarily responsible person in those experiments.

Contribution

(1) Behavioural observation (Paper I)

All experiments I carried out during my Ph.D. studies are based on careful observation of all behaviours performed by the birds during magnetic orientation.

Paper I H. Mouritsen, G. Feenders and I optimised the experimental setup to allow for carefully controlled real time monitoring of the bird's behaviour under different magnetic conditions. In these experiments, a bird was placed inside a cylindrical plexiglas cage with a circular perch, and together with G. Feenders and H. Mouristen, I continuously observed the bird's behaviour in real time on monitors connected to two infrared sensitive video cameras. I developed, set up and optimised the video capturing facilities we used for digitising the video pictures in real time using the software VirtualDub. These digitised video data were required to automatically analyse the **mean orientation** of our birds during magnetic orientation. To further calculate the mean orientation-vector, defined as the position of the bird's head relative to the centre of the cage, I used MatLab routines, custom written by Niko Troje and H. Mouritsen.

Based on the analog video images, we identified a characteristic behaviour besides the well-known migratory restlessness behaviour: stereotype sideways turns of the head that we termed "head scan". Together with G. Feenders, H. Mouritsen and W. Kropp, I quantified specific aspects of the head scanning behaviour, focussing on head versus body orientation preference of the birds during the periods of migratory restlessness.

We found that the birds almost tripled their head scanning frequency in a zero magnetic field, where no magnetic information is available, and the very first directional choice after a head scan in a natural magnetic field leads to an above-chance level of correct directional choices compared to an at-chance level in a zero magnetic field. These findings led to the conclusion that night-migratory birds seem to use head scans to detect the reference direction provided by the ambient magnetic field.

(2) The process of magnetoperception (Paper II, III)

One major focus of my Ph.D. studies was to experimentally test some key theoretical assumptions implied in the model calculations based on magnetoperception via light-sensitive,

radical-pair forming molecules of the retina, acting as chemical sensors (Ritz *et al.* 2000). The first step was to identify and map the distribution of the only class of molecules currently known in vertebrates that is thought to meet the needed biophysical properties. Precisely this is to test if cryptochromes occur in the retina of migratory birds (Paper II) and then to experimentally test their actual biophysical properties and compare these with the assumptions of the model calculation (Paper III).

Paper II Together with H. Mouritsen and G. Feenders, I carefully observed and analysed the bird's behaviour during the crucial 1 hour period prior to tissue collection. I digitised the video pictures in real time using the software VirtualDub, and analysed these digitised data to calculate the mean orientation-vector of the birds during the experimental period of magnetic orientation by means of custom written MatLab routines. Consecutively, H. Mouritsen and I dissected and shock-froze the bird's retinae. Together with P. Dirks, I isolated total RNA from garden warbler retina and developed and carried out the initial identification of garden warbler Cry1a and Cry2. I constructed the full length sequence of garden warbler Cry1a and cloned a 777bp fragment of Cry2. Immunohistochemical analysis of the retina sections indicated that *gw*Cry1a is located in the cytosol of ganglion cells, displaced ganglion cells and in photoreceptor cells, whereas *gw*Cry2 showed clear nuclear localisation. Expression patterns of neuronal activity marker genes such as *c-Fos* and *ZENK* colocalised with *gw*Cry1a in all garden warbler ganglion cells and large displaced ganglion cells at night during magnetic orientation.

Paper III I suggested, planned and mapped out an experimental strategy to test the intrinsic biophysical properties of garden warbler cryptochromes, if they should function as primary magnetoreceptors. In this experimental series, I namely addressed the following key questions: (1) How many members of the cryptochrome multigene family can be identified in the retina of migratory garden warblers? (2) Are cryptochromes of a migratory songbird exited by light? (3) Does the absorption spectrum of the molecule fit the behavioural data? (4) Does the molecule form radical-pairs living long enough (>1µs) to allow a magnetic field effect to take place?

This can be achieved by expressing cryptochromes as holoproteins with their flavin-cofactor inbuilt; I have chosen the baculovirus/*Sf9* cell system to recombinantly express the species-specific proteins in large amounts. I initiated and independently carried out all proceedings in

this respect at the biotechnology company DIARECT AG, Freiburg, who generously provided their equipment and facilities for the entire molecular biological part of this project.

During my Ph.D. studies, I identified four members of the cryptochrome multigene family in the garden warbler (*gw*Cry1a (compare for paper II), *gw*Cry1b, *gw*Cry2 and *gw*Cry4). I subcloned coding regions for *gw*Cry1a, *gw*Cry1b and *gw*Cry1-PHR (PHR = photolyase homology region; a highly conserved N-terminal part of the photolyase/cryptochrome multigene family) into a pVL1392-derived baculovirus transfervector and subsequently expressed the recombinant garden warbler target proteins in a baculovirus/*Sf9* expression system. For selective purification by means of immobilised metal affinity chromatography (IMAC) matrices, I expressed the genes as fusions with a vector-encoded N-terminal $(His)_6$-tag. I identified and isolated positive baculoviral recombinant clones by means of plaque assays and confirmed expression of the recombinant garden warbler cryptochrome by western blot analysis. Biophysical measurements were performed by K. Maeda, K. Henbest & C.R. Timmel (University of Oxford, UK) and E. Schleicher (Freie Universität Berlin, Germany).

I succeeded to express the garden warbler cryptochrome in high purity (*gw*Cry1a, *gw*Cry1b and *gw*Cry1-PHR) with the flavin cofactor built in. This was the first successful recombinant expression of an animal cryptochrome as holoprotein (apoprotein plus flavin cofactor) in a heterologous eukaryotic expression system. By carefully optimising the purification protocol, I overcame the known difficulties (probably due to missfolding of the C-terminal extension) in expression the animal cry-holoprotein and achieved this previously unsuccessful project. In a novel and independently developed strategy, I added an extra on-column denaturation/refolding step to the purification protocol. Successful expression of the holoprotein was the crucial prerequisite for all further biophysical characterisations of the molecule and its putative role in the process of light-mediated magnetoperception, because the suggested radical-pair process is based on the interaction of cry-apoprotein and the flavin cofactor.

We could show that cryptochromes of migratory garden warblers are exited by light. Their absorption spectrum has a range that corresponds to a wavelength range known to elicit magnetic orientation performance in behavioural experiments. Furthermore, we could show that garden warbler cryptochromes form a radical pair intermediate with flavin as a cofactor, and that this intermediate state is very long lived (~ 10 ms), thus potentially allowing magnetic field effects to take place.

(3) Neuronal integration (Paper IV, V, VI)

The method of *behavioural molecular mapping* allows visualisation and linking of brain activity patterns to specific behavioural components. We used this approach to map neuronal activity patterns within the brain, when birds perform magnetic orientation or sit still in breaks between bouts of migratory restlessness behaviour.

In our analyses, we concentrated on the only highly active, sensory, movement-independent part of the entire forebrain that was highly active when night migratory birds perform magnetic orientation. We named this newly identified part of the forebrain of migratory birds "Cluster N" ("N" for night time active) (Paper IV). Based on the first results, we tested expression patterns in Cluster N under various magnetic scenarios, different light conditions and examined for circadian as well as seasonal variance; we also separated the brain input into sensory and motor components (Paper V, VI). In an additional series on European robins, I examined a putative effect of lateralisation in the process of magnetoperception (Paper V).

Paper IV In a close collaboration, Erich D. Jarvis from Duke University, NC, H. Mouritsen, G. Feenders and I aimed to examine the whole brain of migratory and non-migratory birds to detect one or more possible brain regions that are involved in performances, perception and/or integration of magnetic orientation behaviour. We designed a carefully controlled experimental concept that allowed for the first time to get insight into brain areas, active during magnetic orientation in migratory birds. Together with H. Mouritsen and G. Feenders, I carefully observed and critically evaluated the birds' behaviour and sacrificed the birds after they had performed the type of behaviour in focus consistently for a sufficiently long time interval. Under guidance of E.D. Jarvis, G. Feenders and I collaborated on performing preliminary quantitative analyses. We improved the method of quantification, and established and adjusted a standard method for quantification of neuronal activity patterns in night-migratory songbirds. During this work, we focused on the brain area "Cluster N" (N for Night time activation), a newly identified brain area in migratory garden warblers. We could show that Cluster N is not active in non-migratory zebra finches and canaries (*Serinus canaria*). For the first experiments on garden warblers, tested under different magnetic conditions, I did the first anatomical and quantitative characterisations of Cluster N. In our experimental series, we discovered Cluster N as the only highly active, sensory, movement-independent area in the entire forebrain of night-migratory birds performing magnetic

orientation. Comparison of the data I collected on European robins and zebra finch controls with the data from the garden warbler experiment, obtained and processed by G. Feenders, we could prove that Cluster N activity is influenced by light-dependent input through the eye, since covering up the birds' eyes strongly reduced neuronal activity in Cluster N (Paper IV, V).

Paper V To examine a putative effect of light input and/or lateralisation on Cluster N, I designed an experimental series on European robins. I drafted the experimental paradigm and assigned the birds to four treatment groups: monocular occlusion of left or right eye, binocular occlusion of both eyes and binocular controls that received no treatment. To prevent any light from reaching the eye(s) I built small light tight eye cups that were fixed to the bird's head prior to the experiment. Together with G. Feenders, I carefully observed and critically evaluated the birds' behaviour and sacrificed the birds after they had performed the type of behaviour in focus for a sufficiently long time interval. Consecutively, I dissected the brain and shock-froze the hemispheres. For all birds, I sectioned both hemispheres on a cryostat and used radioactive *in situ* hybridisation to visualise the neuronal activity of the brain by means of immediate early genes. I established the behavioural molecular mapping protocol for European robins, a species such experiments have not previously been performed with and processed the brain sections for permanent staining by autoradiography emulsion and Nissl staining. I applied the whole behavioural molecular mapping procedure, from behavioural observation to final analysis of the permanent sections, on the European robins, and zebra finch control birds, with assistance regarding the workload from G. Feenders.

Paper VI While analysing the brain activity patters of garden warblers during night time magnetic orientation (obtained and processed as described for Paper IV) we observed prominent differences in activity levels in several specific brain areas between garden warblers sitting still and those that were moving around in the cage. We correlated the activity levels (quantified by G. Feenders) with the amount of movement behaviour. Number of wing beats, flights, head scans were quantified W. Kropp, G. Feenders and me. We found positive correlation between activity level and amount of movement within 10 cerebral regions and in parts of the cerebellum. Our findings suggest that the motor activated areas are part of a general motor circuit, running in parallel to the well characterised interconnected song nuclei. These findings lead to the formulation of the hypothesis that vocal learning brain systems might have evolved out of a pre-existing motor pathway that controls movement.

Magnetic orientation in migratory birds

– linking behavioural phenomena with underlying molecular and neuronal mechanisms

The sensitivity of animals to the geomagnetic field has proven to be one of the most interesting and lasting challenges in sensory biology. On the occasion of the 125th anniversary of *Science* magazine, the following question has been put forward as one of the 125 biggest questions that face scientific inquiry over the next quarter-century (*Science* (2005), **309**).

*"**How do migrating organisms find their way?** - Birds, butterflies, and whales make annual journeys of thousands of kilometres. They rely on cues such as stars and magnetic fields, but the details remain unclear."*

Animals use a variety of senses and strategies to navigate over distances covering several orders of magnitude. The way an animal perceives the world depends on various environmental constraints and a set of physiological and sensory abilities that differ within and in between species. In the following I will focus on the orientation mechanisms of birds with an emphasis on magnetic compass orientation in night migratory birds.

I. **Introduction** – orientation in migratory birds

***Why* do birds migrate?**

Food, water, protective cover, and a sheltered place to nest and breed are basic to a bird's survival. But the changing seasons at temperate latitudes can transform a comfortable environment into an unlivable one: food and water supply can dwindle or disappear, plant cover can vanish, and competition with other animals may increase. Many animals therefore face the problem of occupying a habitat that is suitable for only a portion of the year. Because of the powers of flight, most birds adapt to seasonal changes in the environment by escaping the area entirely – they migrate. In birds migration is the rule rather than the exception. But how do they know where to go?

I. 1. Spatiotemporal orientation strategies

Box 1 **Orientation and navigation** – Concepts and terminology

It is important to realise that different types of birds do not necessarily use the same orientation strategies (Able 2000). Different species and types of birds have the options and theoretical possibilities to make use of dissimilar navigation strategies. There are at least four fundamentally different spatiotemporal orientation strategies, which may be used by migratory birds: guiding, clock-and-compass orientation, true coordinate navigation and piloting (Mouritsen 2003).

Guiding: Just following experienced conspecifics does not require any orientation or navigation skills (Schüz 1943, 1951; Hochbaum 1955). This strategy implies the presence of experienced "leaders", which themselves use a different strategy. This rather passive orientation strategy is likely to be used by young birds, possessing the instinct to follow adult individuals during a specific time window; in theory many flock migrating birds could use guiding to find their goal area.

Clock-and-compass orientation: This strategy is also often referred to as "vector navigation" (Mayr 1952; Schmidt-König 1965, 1970; Berthold 1991; Mouritsen 1998a, b; Mouritsen & Mouritsen 2000). The bird heads off and orients in a constant inherited compass direction for an innate amount of time. Neither pre-information on route or goal nor any map sense is necessary for this basic form of navigation. Following this strategy, birds do not have an absolute reference system that reliably tells them where they are. This system does not provide feedback, which would enable the bird to correct for directional mistakes stemming from the birds' own navigational system, wind or topography. The time component is controlled for by the circadian clock and/or flight distance. This strategy could in theory be used by both first year migrants and experienced adults.

True coordinate navigation: To determine one's exact geographical position, at least two global coordinates defining a reliable grid and therefore providing a map are necessary (Kramer 1953; Rabøl 1978, 1994; Mouritsen 2001a). Depending on complexity of the strategy, such a map may consist of as little as a coordinate set of the goal. In a more complex version, the bird should have an exact coordinate map of the entire migratory route (Rabøl 1978, 1994). This strategy provides continuous feedback about the bird's current position and therefore allows for direct adjustment of directional mistakes and course corrections en route.

Piloting: This strategy requires previous knowledge about the home area, route and goal, and does not work for inexperienced migrants. Based on visual, auditory, magnetic and/or olfactory landmarks, birds acquire an internal map of the local surrounding (Griffin 1952; Schmidt-König 1965). Following such a sequence of known local landmarks, birds could then find their way by retracing the migratory route.

I. 1.1. *How* do birds know where to go? – Genetic information

To cope with the challenge of migration, it is well established that night-migrating passerines possess an inherent time schedule (Gwinner 1967, 1986, 1996; Berthold 1984; Pulido *et al.* 2001), and at least an inherited initial migratory direction (Berthold 1991; Berthold *et al.* 1992; for a review see Helbig 1996). To find their species- or population-specific winter quarters, night-migrating passerines need to combine the time and spatial information from these prerequisites into a spatiotemporal orientation programme (Mouritsen & Larsen 1998; Mouritsen 1999).

Cross-breeding experiments of blackcaps (*Sylvia atricapilla*) suggest that migratory birds inherit their migratory direction as one component of a "gene package" (Berthold 1999). Besides directional information, the package also includes genes controlling physiological

adaptations necessary for a successful migratory journey, such as temporal component for performance of migratory restlessness, body fat deposition and moult (Berthold & Querner 1981, 1995; Berthold *et al.* 1996). The length of the migratory route seems to be encoded by the bird's circannual clock; the period during which caged migrants show migratory restlessness behaviour is in good accordance with onset, duration and termination of actual migration in wild conspecifics (Berthold 1973, 1975, 1991; Berthold & Querner 1981, 1988, 1995; Berthold *et al.* 1996). Genetically determined migratory direction has been shown to be highly susceptive to microevolution (Berthold *et al.* 1992). Likewise these findings suggest that many if not all bird species have the genetic machinery to migrate and that, with appropriate selection pressures, migratory or sedentary behaviour will become predominant within a given population. A switch to the alternative behaviour can occur in a relatively short number of generations, particularly in population including a continuum of migratory behaviours (Berthold & Helbig 1992; Berthold *et al.* 1992; Berthold & Pulido 1994; Berthold 2000; Outlaw & Voelker 2006).

It is crucial to understand that a strategy that works for one species can not directly be assumed to work for other species, and not all migratory journeys are necessarily the result of the same orientation strategies. The clear difference between the orientation behaviour of first-time migrants and experienced birds was demonstrated in the classic displacement experiment of Perdeck (1958). Both young and experienced European starlings (*Sturnus vulgaris*) were ringed and displaced from the Netherlands to Switzerland. When released, young European starlings on their first migration did not compensate for the displacement. They continued to follow their population specific direction towards south-west for approximately the remaining distance of their original migratory route – resulting in a migratory route parallel to non-displaced control birds. Experienced adults applied a different orientation strategy: they reoriented towards north-west and seemed to be able to navigate back to their traditional population specific wintering sites (Perdeck 1958).

Besides the difference between orientation strategies of first time migrants and experienced adults as demonstrated for European starlings, it is important to realise that different types of birds do not necessarily use the same orientation strategies. *'The challenge of migration'* is not universal, and different types of birds have the options and theoretical possibilities to make use of vastly different navigation systems.

Can we therefore simply generalise Perdeck's impressive results on European starlings (Perdeck 1958) and transfer the observed behaviour to all birds, including night-migrating passerines?

Extreme caution is advisable, because we have to consider that this particular species tested is a day time migrant travelling relatively short distances in large flocks. This scenario does not necessarily require sophisticated orientation abilities of the young birds on their first migration; they just have to follow the old experienced adults and learn where to go *en route* the first time, as has been documented for storks (*Ciconia ciconia*) (Schüz 1950) and many species of waterfowl (Hochbaum 1955). Considering this fact, Mouritsen (1999, 2003) proposed that the vector navigation strategy used by displaced young starlings in Perdeck's study could also be interpreted as an "emergency plan", only activated if a young bird finds itself deserted by adults rather than part of its usual behavioural repertoire.

The scenario described for European starlings significantly differs from migratory behaviour of night-migrating passerines: the young birds on their first autumn migration have to migrate much longer distances at night on their own, without any guidance from adults. One could potentially argue that in the case of night-migrating passerines, selection may have forced them to use a more sophisticated orientation mechanism (Mouritsen 2001b). Consequently, findings such as Perdeck's statement that young European starlings use a simple vector navigation strategy to find their wintering sites cannot be generalised to night-migrating passerines or any other type of bird, until experiments on such birds have verified this finding (Mouritsen 2001b).

I. 1.2. Pigeons – A model organism but not necessarily a universal model

Much research has been conducted on homing pigeons (*Columba livia*) (e.g. Papi 1990; Bingman 1998; Walraff 2001; Wiltschko & Wiltschko 2001a). However, the attempt to transfer knowledge about pigeon homing to orientation strategies of migratory birds should be made with caution.

Pigeon homing and the first autumn migration of young inexperienced birds are two fundamentally different problems; however subsequent migration of experienced adults may be a homing process (Mouritsen 2001a). Per definition, homing is the problem of finding a very well-known location from an unknown location whereas first-time migrants face the problem of finding a completely unknown site from a very familiar area. As a consequence homing

appears to be mainly based on extrapolations of multiple cues from a navigational map (Bingman 1998), whereas the first migration of young birds seems to be based on simple vector navigation with no involvement of a map. Besides, the predecessor of the homing pigeon is the rock pigeon (*Columba livia*), an extreme example of a resident species.

The homing abilities of pigeon stocks raised by humans are to a major degree a result of man-made selection processes and training, which may not have occurred naturally (Mouritsen 2001a). Therefore, the navigational abilities selected for in homing pigeons is not necessarily the same selected for in wild birds by natural selection.

I. 1.3. Young night-migrating passerines – Prerequisites available for orientation

Night-migrating songbirds migrate alone; the adults on average leave the breeding grounds before the juveniles (Mouritsen 2003). They depart on migration flights mostly during or shortly after sunset (Able 1982); the exact time probably differs between species, latitude and season (Åkesson *et al.* 1996; Åkesson & Bäckman 1999).

Inexperienced migrants on their first autumn migration therefore face the problem of finding an unknown species-specific wintering quarter following an unfamiliar migratory route. The only strategies that could potentially be used by young birds travelling on their own are true coordinate navigation or clock-and-compass orientation. Young flock-migrants could use guiding as an additional strategy. Many migratory birds take different routes in spring and autumn (Alerstam 1990, 1996) and therefore one could argue that first year migrants are in similar positions both during spring and during autumn. This is, however, not exactly the case because birds on their first spring migration head towards a known breeding area that they may stored some relevant information about before leaving the area – and this could make a big difference in how they solve the task of finding the correct route (Mouritsen 2003).

I. 2. Orientation tools and compass systems

I. 2.1. How to study migration in a laboratory? – Tools to analyse the phenomenon of migration

The phenomenon of bird migration takes place in mid air and the migratory journeys cover thousands of kilometres. To study and understand the mechanisms of bird orientation and navigation, this global phenomenon has to be brought into the laboratory where the birds' access to suspected orientation cues can be carefully controlled for and selectively manipulated. In the late 1940s, Kramer discovered that caged passerine migrants tend to sit on

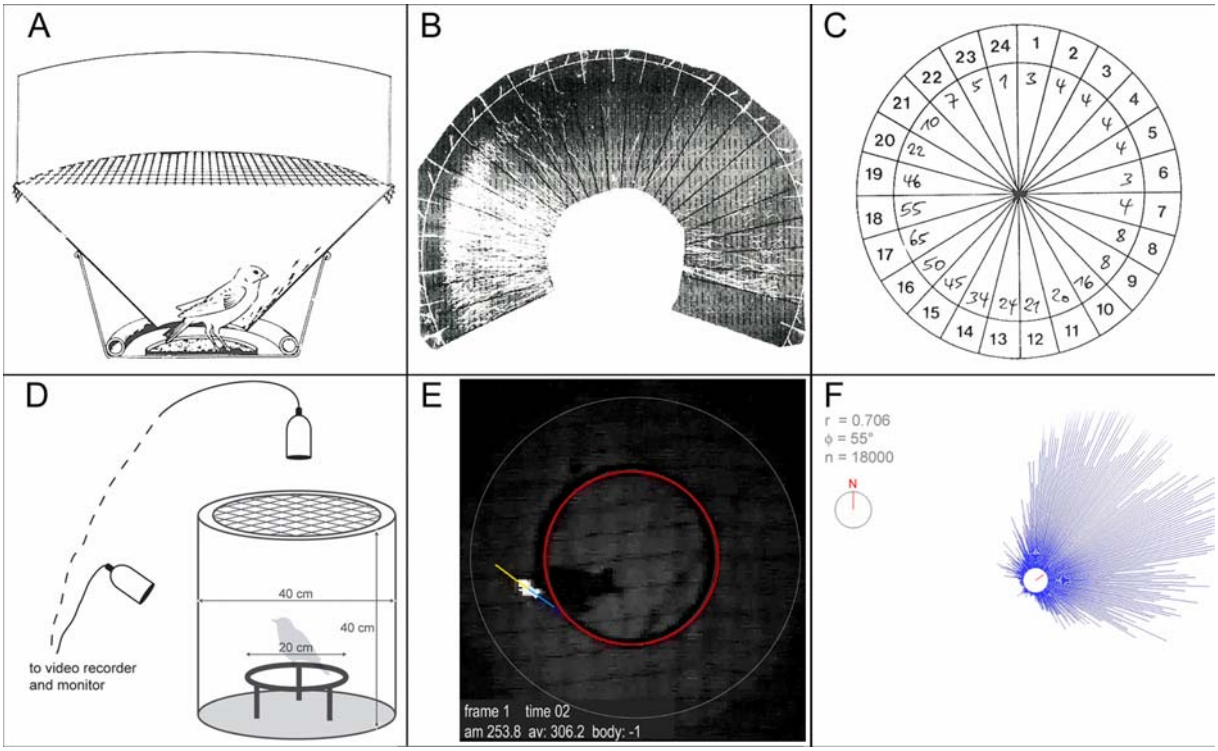

Figure 1: Different types of orientation cages To quantify the orientation preference of caged migratory birds, different types of circular orientation cages have been designed. **A** The original orientation cage was invented by Emlen (Emlen & Emlen 1966) and is referred to as "Emlen funnel". The walls of this funnel-shaped cage were covered with white blotting paper; an ink pad was put at the bottom of the cage. A bird, tested in this experimental setup leaves ink-marks on the cage walls whenever trying to fly in a specific direction. **B** In a slightly altered version, used as standard method in most orientation studies, the ink-pad was removed and the walls were covered with typewriter correction paper (e.g. TippEx) whereon the birds leave scratches whenever touching the cage wall (Rabøl 1979); the picture shows TippEx paper after a 90 min experiment, spread on a light table. **C** Circular diagram showing the distribution of scratches, hand-counted on the paper shown in (**B**). **D** To meet the requirements of experiments aiming to link behaviour to underlying neuronal mechanisms, the cage-design was altered in a way that allows for careful recording of the bird's behaviour: a cylindrical transparent Plexiglas cage is fitted with a circular perch; infrared cameras allow observing birds in real time. **E** Digitised orientation data captured with infrared sensitive cameras [picture shown for camera mounted on top of the cage] allow tracking the position of the bird (reflective tape on the bird's head); outline of the cage and circular perch are highlighted with circles. A custom written MatLab routine tracks position of head-axis and body of the bird [yellow end of line represents rostral part, blue denotes caudal position of the bird's head-axis]. **F** Orientation analysis of a European robin tested in the transparent orientation cage during spring migration under a natural magnetic field. For each experimental session the mean orientation vector with an angle Φ and a length r (varies between 0 and 1) is calculated. The length r can be considered as an inverse analogue of the variance (modified after Berthold 2000; Helbig 1991; Mouritsen *et al.* 2004b).

the perch, flapping their wings and trying to fly or jump into the side of the cage pointing into the direction corresponding to the migratory direction chosen by free-flying conspecifics (Kramer 1949; Emlen & Emlen 1966; Mouritsen 1998b). This characteristic behaviour is called migratory restlessness (*Zugunruhe*) (Kramer 1949). To record and quantify migratory activity and the directedness of the bird's orientation preference during periods of migratory restlessness, several types of orientation cages have been designed over the last decades (Figure 1). The most valuable tool for analysing orientation behaviour in birds was invented by Emlen (Emlen & Emlen 1966) and is usually referred to as the "Emlen funnel" (Figure 1A). The walls of this funnel-shaped cage are covered with white blotting paper and an ink pad is put at the bottom of the cage. In a slightly altered version, which is currently used as the standard method in most orientation studies, the ink-pad is removed and the walls are covered with typewriter correction paper whereon the birds leave scratches whenever they touch the cage wall (Rabøl 1979). In these orientation funnels, inky traces or scratches on typewriter correction paper left by the tested bird on the inclined walls of the cage are quantified to identify the bird's activity level and mean bearing (Figure 1B, C). To meet the requirements of behavioural molecular mapping, which aims to link behaviour with the underlying neuronal mechanisms, we had to modify the orientation-cage design to allow for a precise real-time documentation of the bird's behaviour. In our studies, we therefore designed and used a cylindrical transparent Plexiglas cage fitted with a circular perch and infrared cameras mounted on top and to the side allowing behavioural observation and analysis in real time (Mouritsen *et al.* 2004b, Paper I) (Figure 1D, E, F).

I. 2.2. How are migratory birds able to orient on their migratory journeys? – Compass cues and reference systems

When the analysis of migratory orientation began in the 1950s, the first question concerned the **type of cues** used during migration. Soon after the sun compass (Kramer 1949, 1952), the star compass (Sauer 1957; Emlen 1967a; Wiltschko *et al.* 1987) and the magnetic compass (Merkel & Wiltschko 1965; Wiltschko & Merkel 1966; Wiltschko & Wiltschko 1972) were described for migratory birds. Later, additional external reference factors such as sunset cues, including the pattern of polarised light, were proposed (e.g. Able & Cherry 1986; Able and Able 1990b; Åkesson 1994; Åkesson *et al.* 2001; Cochran *et al.* 2004).

I. 2.2.1. Celestial cues I – the sun

The sun compass was the first orientation mechanism shown to be used by migratory birds (Kramer 1951, 1953). Kramer could show that day-migratory European starlings were only correctly oriented according to their natural migratory direction when the sun was visible. Deflecting the sunlight with mirrors resulted in a corresponding shift of orientation preference (Kramer 1949, 1952). Clock-shift experiments with starlings demonstrated a close link between the sun compass and the birds' circadian clock (Hoffmann 1953b). The sun compass has been shown to be a **time-compensated orientation mechanism** with the relevant compass cue being the azimuth, rather than the sun's elevation for numerous bird species including different taxa (summarised in Wiltschko & Wiltschko 1999).

A second potential orientation cue related to the sun is the polarised light pattern, which has been suggested to be of high relevance for night-migratory birds, especially during the sunset period which usually coincides with the birds' take-off time (e.g. Moore 1982, 1987; Able & Able 1993, 1995; Cochran *et al.* 2004). Information from the sun during the dusk period seem to act as a reference system for night-migratory songbirds to calibrate other compass cues, such as the magnetic field or the stars (Åkesson 1994; Able & Able 1995; Cochran *et al.* 2004).

I. 2.2.2. Celestial cues II – the stars

The first planetarium experiments, suggesting that night-migrating passerines use the stars for orientation, were performed by Sauer (1957). He believed that warblers could truly navigate to their wintering sites with the help of stellar orientation (Sauer 1957). Gwinner and Wiltschko (1978) pointed out that the data collected by Sauer could equally well be explained as effects to an endogenous circannual clock. The definite proof that birds can use the stars for orientation came in 1967, when Emlen published his classical planetarium experiments with indigo buntings *(Passerina cyanea)* suggesting an imprinting-like learning process, which can take place only during a certain sensitive period that ended with the onset of migration (Emlen 1967a, 1967b, 1970). Using a planetarium, Rabøl subjected pied flycatchers (*Ficedula hypoleuca*) and redstarts (*Phoenicurus phoenicurus*) to patterns from different locations on the globe (Rabøl 1998). His results suggest that (in accordance with Sauer 1957) the birds corrected for the simulated displacements and therefore actively navigate. However, neither Emlen (1975) nor Mouritsen and Larsen (2001) saw any signs of true star navigation in their equivalent planetarium experiments.

How to read the stars? In contrast to the geomagnetic field, stellar cues are not stationary and there are several different theoretical possibilities, how birds could use celestial cues for migratory orientation. One hypothesis, first formulated by Sauer (1957), suggests that birds deduce their true geographical position from the rotational phase of the stars. Theoretically, the height of the rotational point of the stars above the horizon could be a measure of latitude and the rotational phase of the stars combined with a dual time-sense could provide longitude information. Birds could potentially use this stellar information to navigate towards a distinct goal travelling along a loxodrome course; another theory proposes true star navigation to be possible by orientation along an orthodrome route[1] (Alerstam *et al.* 2001). An alternative approach to explain the use of celestial cues suggests that night-migrating birds use the stars as part of a time-compensated compass (Schmidt-König *et al.* 1991) but without being able to establish their geographical position from the rotational phase (hour angle) of the stars.

Emlen's investigations (Emlen 1967a, 1967b, 1970), however, strongly suggest that indigo buntings do not make use of the rotational phase of the starry sky, neither to identify geographical position nor as part of a time-compensated star compass (working in a similar manner as the sun compass). Instead, his birds seemed to use only the location of the rotational point and subsequently the constant geometrical pattern of the stars to define geographical north. Emlen could further show that hand-raised birds learned the position of the centre of rotation during ontogeny independent of which star was positioned at the rotational point (Emlen 1972, 1975). Hand-raised birds that had not seen the starry sky (or any artificial light dot substitute) before onset of migration did not orient. Even subsequent exposure to the natural sky did not allow for star-based orientation (Emlen 1969).

Once, the birds start migration, they seem able to deduce geographical north from the geometrical configurations of the stars independently of current stellar rotation (Emlen 1975), that is the birds use the stars for **time-independent celestial compass** orientation. This hypothesis is consistent with the orientation behaviour observed in subsequent experiments carried out by Wiltschko and co-workers (Wiltschko *et al.* 1987). They hand raised garden warblers (*Sylvia borin*) in four different parts of one room, where they experienced an artificial sky simulated by a set of diodes rotating around the centre of the room. Depending on the position in the room, birds of the four groups experienced the centre of rotation to be located at different directions. In subsequent orientation experiments, they oriented in different

[1] A *loxodrome* course is equivalent to a constant compass course route. An *orthodrome* route, however, describes orientation along the Earth's great-circle routes, representing the shortest distance between two points on the globe.

directions, consistent with an innate strategy, which told them to migrate "*away from the learned centre of rotation*" (Wiltschko *et al.* 1987). This was the direct proof that the birds perceived the artificial light dots as "starry sky" and learned the geometrical star configurations to pinpoint and calibrate the rotational point of the "starry sky" as geographical north. This finding could be confirmed by a number of following studies (e.g. Able & Able 1990a; Mouritsen & Larsen 2001), which strongly suggest that birds do not inherit detailed knowledge about the star pattern, but they are equipped with an inherited strategy to look for the centre of rotation and interpret this as "polewards".

To conclude, the data presented (Emlen 1967a, 1967b, 1970, 1972, 1975; Wiltschko *et al.* 1987; Able & Able 1990a; Mouritsen & Larsen 2001) are consistent with the suggestion that night-migrating songbirds are unable to deduce geographical longitudinal position (west-east) from the stars. To detect longitudinal position the birds would need to make use of two clocks, one of which should be very accurately fixed on home-time (Mouritsen & Larsen 2001). Gwinner has convincingly demonstrated that birds have an internal clock that quickly adapts to local time (Gwinner 1996; Gwinner *et al.* 1997); however, no evidence of a fixed-time internal clock has been found in any species.

I. 2.2.3. Geomagnetic compass

Birds keep on migrating when the sky is covered with clouds and the star compass therefore would be of no use. In this weather condition, night migratory birds only have the option to orient according to the Earth's magnetic field. To a first approximation, the Earth's magnetic field resembles the dipole field of a giant bar magnet with field lines leaving the southern hemisphere, curving around the globe and re-entering the planet in the northern hemisphere and therefore presents an omnipresent reliable source of reference compass information (Figure 2). The *polarity* of the field lines (determined by the horizontal component of the field lines) points from south to north anywhere on Earth. *Inclination* (defined by the angle between the geomagnetic field lines and the Earth's surface/gravity) changes with latitude. They perpendicularly leave the Earth's surface at the magnetic South Pole, run parallel at the magnetic equator and perpendicularly re-enter the Earth at the magnetic North Pole. The magnetic field *intensity* also changes with latitude: it is weakest at the equator (~30.000 nT) and gradually increases towards the magnetic poles (~60.000 nT). Thus, several geomagnetic parameters, such as polarity, inclination angle and field intensity, vary across the planet's

surface in a manner that even makes them at least partly suitable for use in a position-finding, i.e. map sense (e.g. Lohmann *et al.* 1999).

The hypothesis that migratory birds utilise the geomagnetic field for orientation has been proposed as early as 1859 (von Middendorff 1859), and the use of a magnetic compass in night-migrating passerines was first demonstrated for the European robin (*Erithacus rubecula*) in 1965, taking advantage of the spontaneous behaviour of migratory restlessness (Merkel & Wiltschko 1965; Wiltschko & Merkel 1966; Wiltschko 1968; Wiltschko & Wiltschko 1972). Meanwhile, magnetic compass orientation has been described for a variety of other bird species (Wiltschko & Wiltschko 1996), such as several passerine migrants (e.g. the garden warbler: Wiltschko 1974), homing pigeons (Walcott & Green 1974) and a shorebird species (Gudmundsson & Sandberg 2000). The functional mode of the magnetic compass was first demonstrated in birds. Further behavioural experiments have revealed several characteristic properties of the magnetic compass of birds.

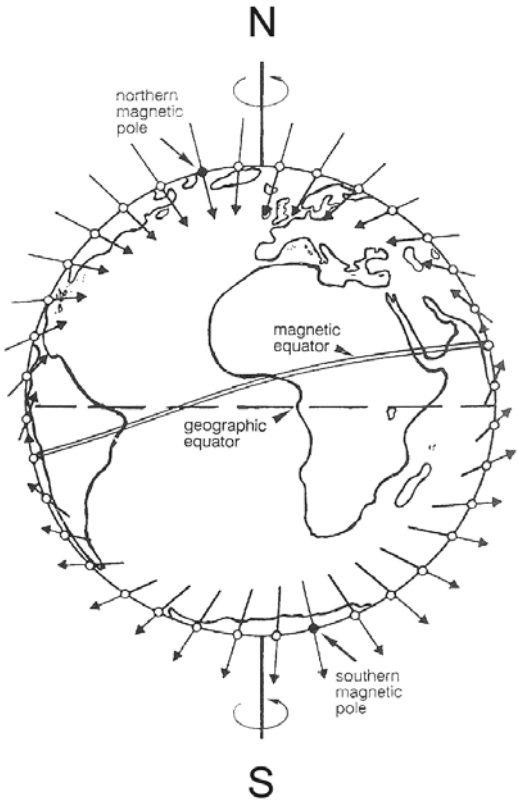

Figure 2: The Earth's magnetic field The arrows indicate the direction of the magnetic field lines. Their length is drawn relative to the magnetic field intensity at different latitudes. The steepness of the magnetic field lines relative to the surface of the Earth shows the angle of inclination (from Wiltschko & Wiltschko 1996).

In all bird species studied so far, including short- and long-distance migrants, birds of both hemispheres, night, twilight and day migrants (Wiltschko & Wiltschko 1996), the magnetic compass was shown to be an **inclination compass** – sensitive to the axis but not to the polarity of the magnetic field lines (Wiltschko & Wiltschko 1972). This is in contrast to the technical compass humans use to orient. Our *polarity* compass uses the horizontal component of the field lines as a reference, which reliably faces from south to north everywhere on the planet, regardless of the hemisphere. The avian *inclination* compass, however, provides the animals with information about the axial alignment of the magnetic field but does not perceive the polarity (direction) of the magnetic field lines. Thus, an inclination compass separates beween equatorwards and polewards but not between north and south. Transequatorial migrants therefore face two problems: (1) the inclination compass is bimodal in the horizontal field

around the magnetic equator, and (2) beyond this zone, transequatorial migrants must reverse their migratory direction with respect to their inclination compass to continue in the same geographical direction (e.g. the former strategy "*fly towards the equator*" has to switch to "*fly towards the pole*" to stay on the right track). Experiments on garden warblers indicate that the horizontal field itself may serve as a trigger and cause changing migratory direction from *equatorwards* to *polewards* (Wiltschko & Wiltschko 1992; Beason 1992). This leaves the problem of the specific situation at the magnetic equator. Garden warblers were disoriented when a horizontal magnetic field was the only available orientation cue (Wiltschko 1974). However, in nature, additional factors such as the stars or sunset cues may help the birds to master the situation (Beason 1992). Experiments on free flying *Catharus* thrushes suggest that the magnetic compass is calibrated daily by sunset cues during twilight (Cochran *et al.* 2004). The direction of sunset-related twilight-cues would allow for a correct interpretation of the magnetic field, even around the equator, where the magnetic compass on its own is difficult to use.

Another noteworthy feature of the birds' magnetic compass is that it is narrowly tuned to the total intensity of the ambient magnetic field (Wiltschko & Wiltschko 1972, 2001b; Wiltschko *et al.* 1978). Robins captured and kept in a local magnetic field were able to orient only within a narrow intensity window (local field intensity ± 25 %), but were disoriented under field intensities outside this intensity-range. However, after being housed three days in fields with different intensity, birds were able to adapt to changes in field strength exceeding those experienced during natural migration (~30.000 nT at the magnetic equator to ~60.000 nT at the magnetic poles) (Wiltschko & Wiltschko 1978). Recent findings indicate that the time period required to adjust to magnetic intensities, which they cannot spontaneously use for orientation is significantly shorter than the three days previously tested (Wiltschko *et al.* 2006): European robins are able to orient under magnetic field conditions, twice as strong as the local geomagnetic field at the normal testing site, after merely 1 hour of preexposure (at least in birds that had previously experienced this field). After adaptation to new intensity windows, the birds still show correct orientation under the previously experienced intensity range but they are not able to inter- or extrapolate and orient under intermediate intensities not experienced before. In the context of natural migration, intensity-adaptability of the magnetic compass is a key prerequisite for reliable magnetic compass orientation. Migratory birds move from northern regions with rather high magnetic field intensities (~50.000 nT) to their wintering quarters in the south where they experience considerably weaker local fields (~30.000 nT). Thus, the magnetic compass should allow for adjustment to gradually changing

magnetic intensities (*decreasing* intensities on their southward journey in autumn and *increasing* during spring migration).

Behavioural experiments have shown that magnetic orientation performance under low-intensity monochromatic light is dependent on the wavelength of the ambient light. Birds orient well in blue and green, but not in yellow or red light (Wiltschko *et al.* 1993, 2001a; Wiltschko & Wiltschko 1995, 1999, 2001b, 2002; Muheim *et al.* 2002; Munro *et al.* 1997a, Rappl *et al*. 2000) (Figure 3). Experiments, trying to elucidate the effect of higher intensities or a mixture of colours of monochromatic light suggest a rather complex relationship between the receptors involved in magnetoreception: birds show responses that are not in line with the expected migratory direction and are in some cases bipolar (Wiltschko *et al.* 2000, 2003a, 2004a, 2004b). One speculative explanation of these findings is that two different magnetosensitive mechanisms interact under this rather unnatural scenario: one dominant light-dependent mechanism that requires light of the blue-green spectral range and a secondary mechanism operating under long-wavelength light or maybe not requiring any light at all (Wiltschko *et al.* 2004a, 2004b).

Lateralised dominance of brain hemispheres is a common phenomenon in birds and other animals. In birds, asymmetries of visual functions are well studied, with each hemisphere being specialised for different tasks (Rogers 1996). The visual system of birds seems to be split into a left-eye system predominantly responding to detailed properties of a given stimulus (e.g. Deng & Rogers 1997), and a right-eye system, which is mainly responsible for further categorisation of the stimulus (e.g. Clayton 1993; Clayton & Krebs 1993). In 2002, Wiltschko and colleagues reported lateralised functions of the birds' visual system associated with magnetoperception

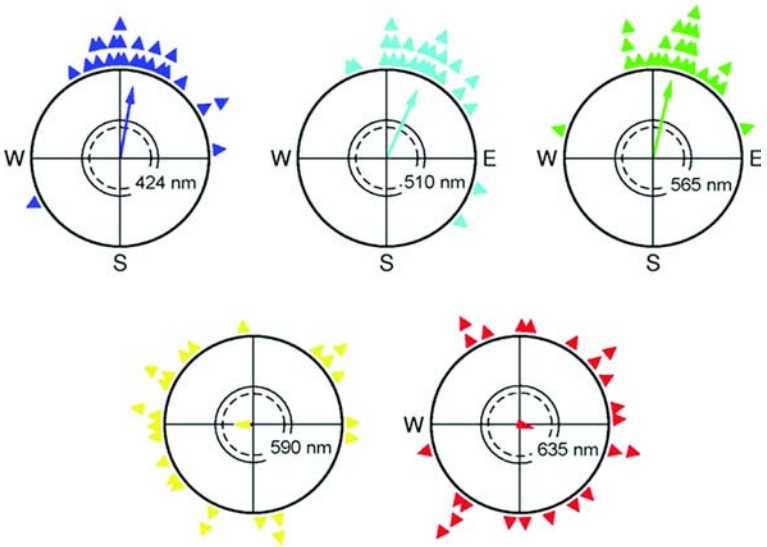

Figure 3: The magnetic compass of birds is wavelength dependent European robins were tested under different wavelength during spring migration: birds tested under monochromatic blue, turquoise and green light show seasonally correct northwesterly magnetic orientation. Under light of the yellow or red spectral range however, the birds were disoriented. Each triangle represents the mean orientation vector of one single bird. The arrow represents the group mean orientation vector (*r*) (modified after Wiltschko & Wiltschko 2002).

(Wiltschko *et al.* 2002a). European robins tested with the magnetic field as the only available orientation cue were well oriented in their natural migratory direction when using their right eye solely, but failed to show a significant directional preference when only receiving visual input via their left eye (Wiltschko *et al.* 2002a). Comparable experiments with Australian silvereyes (*Zosterops lateralis*) resulted in the same conclusion (Wiltschko *et al.* 2003b). These findings suggest a marked dominance of the right eye/left brain hemisphere in the process of magnetoperception for magnetic compass orientation.

Taken together, these findings indicate that reception of magnetic compass information is light-dependent. Furthermore, birds with their pineal gland[2] removed are still able to use their magnetic compass to orient in the lab when receiving injections of melatonin (Schneider *et al.* 1994). This finding further strengthens the suggestion that the eye is somehow involved in the process of detecting magnetic compass information in birds whereas the pineal gland is at least not crucial for magnetic compass orientation.

I. 2.2.4. Hierarchy – interaction of different compass mechanisms

Many bird species were found to use more than one type of reference compass system and the focus of study rapidly shifted to questions about the hierarchy. Despite the large number of cue-conflict experiments, our current understanding of the relative importance of different compass cues does not allow for an overall hierarchical rating. Thus, the relative importance of the different compass orientation cues remains controversial (e.g. Moore 1987; Åkesson 1994; Wiltschko *et al.* 1998b). The ambiguous and controversial results have been extensively reviewed (see e.g. Able 1993; Åkesson *et al.* 2002; Muheim *et al.* 2006) and were mainly interpreted to be due to high plasticity in the relative importance of compass cues both between species (Gwinner & Wiltschko 1978; Helbig *et al.* 1989) and during ontogeny. It may also be the case that birds apply different cue-calibration strategies depending on the ecological scenario they are facing (Moore 1985; Sandberg & Moore 1996; Cochran *et al.*2004). One major problem in interpreting the literature on cue-conflict experiments, however, seems to be the lack of standardised holding conditions, as well as variable experimental paradigms and procedures that complicate a direct comparison between different experimental results.

[2] Several converging lines of evidence point to a role of the pineal complex of amphibians in magnetoreception (summarised in Deutschlander *et al.* 1999).

To understand the relative importance and interrelationships among the different compass systems, three main complementary approaches were used, namely: (1) exposing hand-raised birds to manipulated putative orientation cues during the pre-migratory period and subsequent testing for possible consequences on the birds' orientation during their first autumn migration (e.g. Bingman 1983; Prinz & Wiltschko 1992; Able & Able 1993; Weindler & Liepa 1999); (2) testing the response of wild caught birds, when suddenly confronted with conflicting cues (e.g. Bingman 1984; Able & Able 1997; Weindler & Liepa 1999; Prinz & Wiltschko 1992; Wiltschko *et al.* 1998a), and (3) exposing birds to a cue-conflict during the migratory season and subsequent testing in the absence of the cue-conflict (e.g. Wiltschko *et al.* 1998b, 2001b; Sandberg *et al*. 2000; Åkesson *et al.* 2002; Cochran *et al*. 2004). The experimental procedures were mainly based on analyses of behaviour in caged migrants (but also radio telemetry e.g. Åkesson *et al.* 2001), since this provides the best possibilities to specifically manipulate potential orientation cues and to create test situations in which the various cues gave conflicting information.

The ontogeny of orientation mechanisms in migratory birds seems to involve a complex of programmed learning rules and calibrations between the several interacting compasses used for navigation. Cue-conflict experiments seem to indicate two distinct phases of migratory orientation in which the magnetic and celestial reference systems interact in different ways (Wiltschko *et al.* 1998a). These two phases are the premigratory period and the time during migration (for a recent review see Muheim *et al.* 2006).

The premigratory period involves the conversion of genetically encoded information into an actual compass course. Celestial rotation alone provides a reference direction "away from the rotation centre" (on the northern hemisphere corresponding to geographic south) leading towards the equator. Species and population-specific deviations from this reference direction seem to be coded only with respect to the geomagnetic field (Gwinner & Wiltschko 1978); and apparently information from celestial rotation has to be linked to information from the geomagnetic field to establish a population-specific direction with respect to the stars. Analyses suggest that during the premigratory phase, celestial information (stars and/or sunset cues) dominates over other orientation cues and especially sunset cues (which may be or include polarised light patterns from the region of the sky near the horizon) appear to provide the calibration reference for the magnetic compass, both in juveniles and experienced adults (Bingman 1983; Prinz & Wiltschko 1992; Weindler & Liepa 1999; Cochran *et al.* 2004).

Once the initial course is set the actual orientation during migration begins. As already outlined above, night-migrating birds seem to use both celestial cues and the Earth's magnetic field as reference systems, but in the migratory phase in contrast to the premigratory period, the majority of experiments suggest that birds primarily rely on their magnetic compass and use it to calibrate celestial compass cues (Wiltschko & Wiltschko 1975a, 1975b; Wiltschko *et al.* 2001b; Sandberg *et al.* 2000, 2002). In most, but not all cue-conflict experiments, the stellar constellation is calibrated to be in agreement with directional significance of the ambient magnetic field (Wiltschko & Wiltschko 1975a, 1975b, 1976; Beason 1987; Bingman 1987; Weindler *et al.* 1998; Sandberg *et al.* 1988, 2000; Sandberg & Pettersson 1996; Able & Able 1996, 1999) suggesting a reversed rating of celestial and magnetic cues compared to the premigratory period. Conflict experiments on birds of the northern and southern hemisphere, however, indicate a certain asymmetry. Results of these experiments propose the potential importance of the direction of celestial rotation, which seems to influence compensatory performance (Wiltschko & Wiltschko 1998).

Why does the relative importance change? Given the complex interactions between different compass systems, there can not be one simple answer to this question. One possible reason may be the geographical variation of the reference systems themselves: The pattern of celestial rotation undergoes a striking change due to latitudinal shift during migration – familiar stars disappear below the horizon and new ones appear that have to be calibrated. On the other hand, the geomagnetic field becomes an increasingly regular, reliable factor that is available irrespective of weather conditions.

Recent orientation experiments carried out on free-flying *Catharus* thrushes in the wild suggest that they primarily use a magnetic compass in midair (Cochran *et al.* 2004). The night-migratory songbirds experienced a shifted magnetic field during the sunset hours in an outdoor cage. After exposure to the changed magnetic field, the birds were released with a radio transmitter attached and followed by radio telemetry. The headings of the birds were in close agreement with the magnetic mirror-image directions predicted if the birds had calibrated their magnetic compass from sun-based twilight cues (Cochran *et al.* 2004). In the subsequent night, the thrushes were shown to recalibrate their compass direction again before departure. The experiment clearly shows that the thrushes calibrated their magnetic compass according to sunset cues (with direct reference to the sun and/or the polarised skylight pattern).

What we can learn from the cue-conflict literature is that, as researcher working in the field of bird orientation, we have to be extremely cautious and keep in mind that any artificial experimental situation may be very sensitive to small changes in the experimental setup and the relative preference of one cue or the other may therefore not necessarily reflect the natural choice of the bird. It is important to realise that experiments, which seem to look identical at a first glance may substantially differ regarding the overall experimental paradigm. Main variables regard (i) the **experimental bird**: species and age (experienced versus first migrants); (ii) the **holding conditions**: cages housed indoors versus outdoor aviaries, exposure to the celestial cues or not; and (iii) the **testing condition**: experimental season, place (capturing and testing location), with regard to a time-shift, there are two variables to be considered namely direction of shift (clockwise or counterclockwise) and dimension of shift, cues available during the cue-conflict.

Standardised experimental designs and experimental paradigms that allow for direct comparison as well as new approaches, relying on the behaviour of free flying birds (Cochran *et al.* 2004), are necessary to get a better understanding of interrelation and importance of the different compass systems.

It may also be the case that the varying dominance in compass cues one finds when reviewing the literature in fact indicates that under natural conditions celestial cues and the magnetic field should not be regarded as two alternatives, but as integrated components of a complex mechanism for direction finding that is based on both cues alike. However, it has been shown repeatedly that migratory birds can orient based on each one of these cues alone, when the other cues are experimentally removed (e.g. Mouritsen 1998a).

To sum up, this chapter has outlined that behavioural experiments have led to a fairly good understanding of the compass systems that enable migratory birds to find their way over thousands of kilometres. During my Ph.D. studies, I mainly focussed on the cognitive, physiological and molecular mechanisms, underlying magnetic orientation in night-migratory songbirds. Results of this integrative approach are presented in the following chapters, focussing on the receptor level and the neuronal processing and integration level, respectively.

II. **Magnetic compass orientation in birds** – detection

II. 1. **Background**: Magnetoperception mechanisms

Currently – even though the role of the magnetic compass in the orientation of birds is fairly well understood – the physiological mechanism(s) enabling birds to sense the Earth's magnetic field remain the only major unexplained sensory modality in biology. Whereas receptors for most other sensory systems have been characterised and studied in detail, neither the primary receptor molecule(s) nor reception mechanism(s) underlying magnetic field detection have yet been identified with certainty in any animal. As outlined above, most of what is known about magnetoreception has been inferred from behavioural experiments, theoretical considerations and few anatomical and electrophysiological studies.

Why is it so difficult to locate magnetoreceptors? The Earth's magnetic field is difficult to perceive if only biological material is available as potential receptor. The magnetic fields passes freely through biological tissue and therefore the sensory organ(s) can potentially be located anywhere in the body. They do not have to directly contact the external environment to detect stimuli as other sensory organs do.

Many hypotheses have been proposed suggesting how birds may potentially sense the Earth's magnetic field (e.g. Leask 1977; Schulten *et al.* 1978; Schulten 1982; Edmonds 1996; Liboff *et al.* 2000; Ritz *et al.* 2000; Kirschvink *et al*. 2001). Two biophysical mechanisms have since emerged as most promising for magnetodetection (e.g. Mouritsen & Ritz 2005): magnetite-mediated magnetoreception (Beason & Semm 1987, 1996; Semm & Beason 1990; Fleissner *et al.* 2003) and light dependent chemical magnetoreception (Schulten *et al.* 1978; Ritz *et al.* 2000, 2004).

II. 1.1. Magnetite

Behavioural and electrophysiological evidence in favour of a magnetite-based magnetoperception mechanism have been obtained in fish (Walker *et al.* 1997). Magnetite-based magnetoreceptors based on freely rotating single-domain magnetite particles (Kirschvink & Gould 1981), fixed superparamagnetic (SPM) magnetite particles (Kirschvink *et al.* 1985; Shcherbakov & Winklhofer 1999; Davila *et al.* 2003; Fleissner *et al.* 2003) or magnetite-containing liquid crystals (Edmonds 1996) have been suggested. Magnetite crystals act like a compass needle and enable magnetotactic bacteria to passively align to the geomagnetic field

(Blakemore 1975). Magnetite crystals have been found in many animals, but the sole presence of magnetite in any organism is the rule rather than the exception; magnetite synthesis seems to be a general way for organisms to deposit excess iron. To be potentially relevant for the process of magnetoreception, magnetite-rich structures have to be connected to nerve fibres. Otherwise, they cannot provide magnetic information for integration and processing in the brain.

In birds, magnetite containing candidate structures in the upper beak of pigeons have recently been characterised on an ultra-structural scale (Fleissner *et al.* 2003); the magnetite in this receptor structure is of superparamagnetic nature. The clustered structures are arranged in groups of 10-15 clusters of magnetite crystals at three fixed locations along each margin of the bird's upper, inner beak (Figure 4). These highly ordered groups of magnetite clusters are embedded in a dendrite ending of an unmyelinated neuron. In addition, noncrystalline platelets seemingly made of iron phosphate occur in the same cell types and are discussed as putative geomagnetic field enhancers, which might concentrate the magnetic field toward the site of the superparamagnetic clusters. These findings indicate a direct connection of the magnetite structures to nerve fibres that access the brain via the trigeminal nerve (Fleissner *et al.* 2003). Biophysical characterisation of the putative magnetite receptor structure now opens the field for theoretical modelling and may allow insight into more accurate predictions and interpretations of effects on

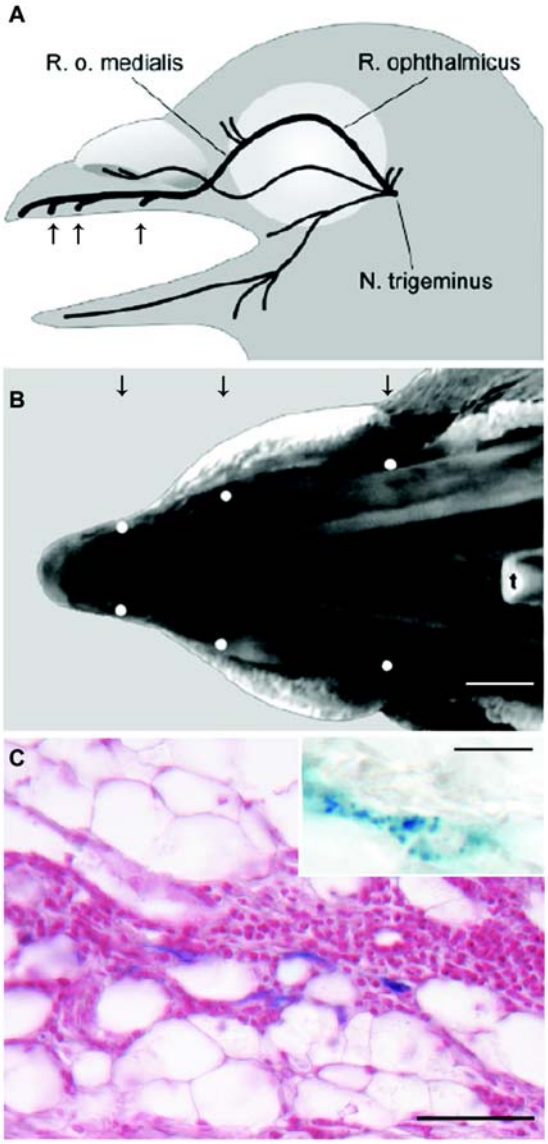

Figure 4: Localisation of putative magnetoreceptors in the beak of homing pigeons **A** Schematic drawing of the pigeon skull. As indicated by arrows, the tip of the upper beak gets somatosensory innervation by the ophthalmic branch of the trigeminal nerve (median branch: R. o. medialis = ramus ophthalmicus medialis). **B** Macroscopic view of the interior of the upper beak. The sites of the candidate magnetoreceptor nerve endings as derived from serial histological sections are indicated by arrows and white dots (t, tongue). **C** Prussian Blue (PB) stained section of the skin of the upper beak of the homing pigeon. PB-reactive, high concentrations of iron appear as small dense spherules and pale-blue background staining. An area of about 250 µm in diameter shows multiple labelled nerve endings. The stack reconstruction in the right upper corner shows marked nerve ending containing a chain of dark-blue spherules. Scale bars 2 mm in **B**, 50 µm in **C** (main figure) and 10 µm for the reconstruction (modified after Fleissner *et al.* 2003).

orientation performance of strong permanent magnets (Mouritsen *et al.* 2003; Bonadonna *et al.* 2003, 2005) and strong magnetic pulses (Wiltschko *et al.* 2002b; Davila *et al.* 2005).

A putative magnetite-based magnetosensor in birds may be tuned to sensing small differences in magnetic field intensity and might be involved in a magnetic **map** or **signpost sense**[3], which might enable them to approximate the direction of and perhaps distance to home from distant and unfamiliar locations (Munro *et al.* 1997b; Mouritsen & Ritz 2005), rather than a **magnetic compass** (Wiltschko & Wiltschko 1995).

Recent operant conditioning experiments have demonstrated that pigeons can discriminate between the presence and absence of a strong magnetic anomaly (~100.000 nT) (Mora *et al.* 2004). This discrimination-ability is destroyed when the ophthalmic branch of the trigeminal nerve was lesioned, but remained when the olfactory nerve was sectioned (Mora *et al.* 2004). These findings strongly support the hypothesis of an involvement of the trigeminal nerve in processing magnetic information from the beak to the brain (Beason & Semm 1996). Magnetic compass orientation responses (selecting and maintaining a direction) appear not to be affected by blocking the ophthalmic branch of the trigeminal nerve (Beason & Semm 1996). These findings are in line with the suggestion that the magnetite structures located in the bird's beak detect magnetic intensities, which could in turn potentially work as a magnetic "map-" or "signpost"-sense. However, in order for a magnetic "map-sense" to work, birds must be able to detect naturally occurring local changes in magnetic field strengths that are about five orders of magnitude smaller (~10 nT) than the magnetic anomalies used in the operant conditioning by Mora *et al.* (2004). Therefore, to function as a putative biologically relevant "map-sense", it still needs to be demonstrated that the magnetite-based trigeminal magnetodetection system can detect biologically relevant magnetic anomalies (Mouritsen & Ritz 2005).

II. 1.2. Chemical sensor

In 1978, Schulten proposed that magnetic sensing might have spin chemical origins (Schulten *et al.* 1978; Schulten 1982). He suggested that the yield of a biochemical reaction proceeding via a radical pair might be sensitive to the orientation of an external magnetic field. Earth strength magnetic fields influence correlated spin states of paired radicals because of anisotropy in the hyperfine interactions (Schulten 1982; Ritz *et al.* 2000; Weaver *et al.* 2000).

[3] A magnetic **map sense** usually implies that birds can sense magnetic intensity in the order of 10 nT, whereas a magnetic **signpost sense** implies that birds can recognise large changes in intensity and/or inclination and use this as a sign to change one or more aspects of their behaviour, such as fattening (e.g. Fransson *et al.* 2001).

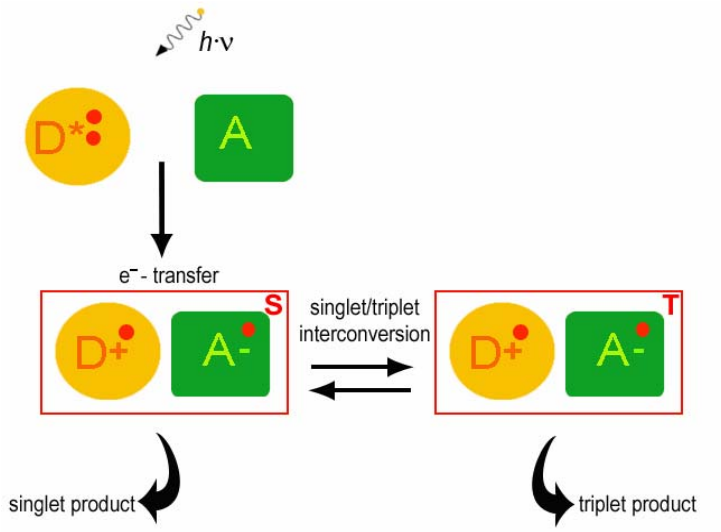

Figure 5: Reaction scheme for a radical pair based magnetic compass The putative process starts with an electron transfer from a donor molecule **D** to an acceptor molecule **A** upon photo excitation, resulting in a radical pair with both donor and acceptor molecule possessing one unpaired electron. The spins of the unpaired electrons can be either opposite (singlet state, *S*) or parallel (triplet state, *T*) and both states can interconvert. An external magnetic field affects the interconversion between singlet and triplet intermediates, and therefore modulates the ratio of singlet and triplet states depending on the orientation of the molecule within the field. In a final step, singlet and triplet intermediates will react to distinct products, differing in their biochemical properties (modified after Ritz *et al.* 2000).

Electron spins are not strongly coupled to the thermal bath and therefore represent one of only a few molecular features that might plausibly be influenced by the Earth's magnetic field (Edmonds 2001). The suggested mechanism (Figure 5) involves a light-induced electron transfer between two molecules. The electron transfer results in the generation of a radical pair intermediate that will either exist in a singlet or a triplet excited state, and subsequently decay in chemically different singlet or triplet products. Theoretical calculations (Eichwald & Walleczek 1996; Ritz *et al.* 2000) and *in vitro* experiments (Harkins & Grissom 1994, 1995) showed that the ratio between singlet and triplet products from radical-pair reactions can be modulated by an Earth-strength magnetic field, thereby potentially providing the basis for a magnetic compass. This proposal has been revived more recently by Ritz *et al.* (2000), who proposed that the retina with its almost perfect half-ball shape is well suited as an ordered structure, and that the radical pair intermediate is involved in some kind of visual reception system. According to this theory, the reaction yield anisotropy of the receptor radical pair governs the directional response.

Assumed prerequisites in the model calculations

For a spin-chemical receptor mechanism to detect the direction of magnetic fields as weak as that of the Earth's, several stringent conditions must be met (Grissom 1995; Adair 2000; Ritz *et al.* 2000; Weaver *et al.* 2000; Mouritsen *et al.* 2004a, Paper II): (**1**) A molecule fulfilling these requirements must **exist** in the retina of migratory birds; (**2**) the cells containing the candidate molecule have to be **active at night**, when the bird performs a magnetic orientation task; (**3**) **motion** of the radical pair forming molecule has to be restricted, i.e. the molecule must somehow be fixed in the cell. (**4**) The molecule has to be **excited by light**, and (**5**) the **absorption spectrum** has to match the range that has been shown to elicit magnetic orientation

performance in behavioural experiments. (**6**) The **half-life time** of the light-excited radical-pair intermediates must be **long enough** (> 1 µs) that they can be differentially affected depending on the direction of Earth's strength magnetic field. (**7**) Because the influence of the Earth's magnetic field on the putative chemical processes is rather weak, the effect must presumably be **integrated over a large area**, and (**8**) a suitably **sensitive transduction** of the changing product yields is necessary (e.g. one reaction product might be a neurotransmitter, the other not or one end-product interfering with the sensitivity of a visual receptor, the other not etc.). (**9**) A further crucial requirement is that the initial electron transfer must not randomise the original parallel or opposite **spin relationship** of the two electrons. This is not true for all electron transfer processes but is often the case when the transfer is induced by photo-excitation. These considerations together with the data from behavioural experiments (e.g. Wiltschko & Wiltschko 2001b) have led to the suggestion that the putative chemical magnetoreceptor might also be photoreceptive (Ritz *et al.* 2000) and should be located in the eye; in this case a link via photopigments to the visual transduction system might be used for transduction, i.e. birds may "see" the Earth's magnetic field.

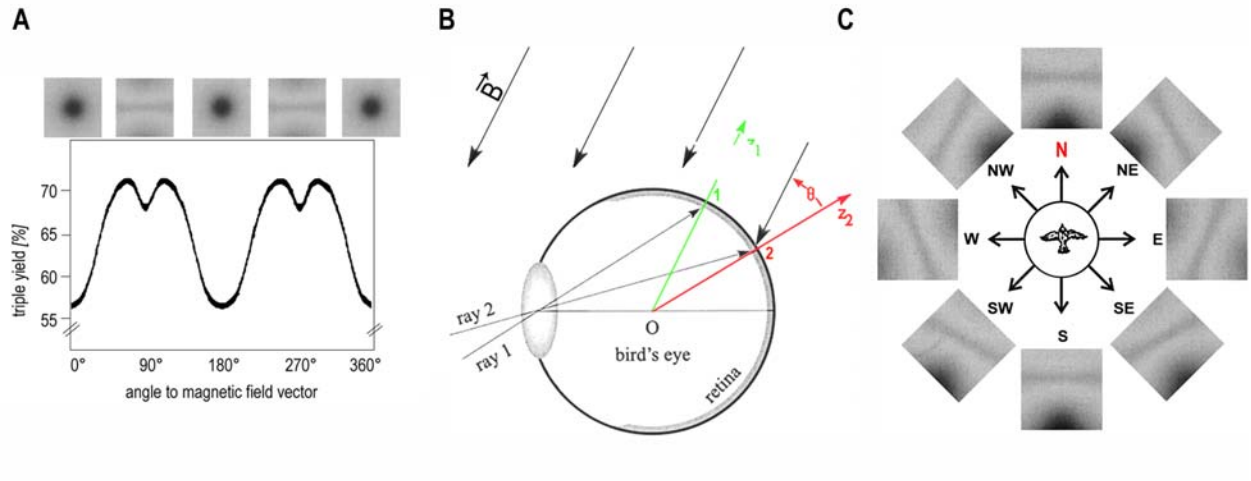

Figure 6: Light mediated magnetic compass orientation in birds Assuming a fixed orientation of a radical pair forming photopigment in the bird's eye, the magnetic field would modulate the radical pair reaction and thereby affect light signalling differently in different parts of the retina, leading to perception of the ambient magnetic field as a modulated visual pattern. **A** The graph depicts the axial response of the triplet yield of the radical pair reaction from the angle of the radical pair to the external magnetic field. Depending on the orientation of the bird within the field, the perceived visual patterns (sketched in the top row) vary and therefore potentially allow for encoding of directional information. The pattern-modulation does not encode information about polarity; it repeats itself every 180°. **B** Ideal model of the bird's retina: the receptor molecules are assumed to be oriented normally to the sphere of the half-ball shaped retina (directions z_1, z_2). Depending on the molecule's location on the retina, the magnetic field vector is perceived at different angles (θ). Integration over the entire retina would potentially enable the bird to sense the directional information provided by the Earth's magnetic field via the visual pathway. **C** Visual modulation patterns through the geomagnetic field as it would be perceived by a bird, flying parallel to the horizon (geomagnetic field inclination 68°, 50 µT) and looking towards N, NE, E, SE, S, SW, W and NW (modified after Ritz *et al.* 2000).

Assuming the necessary fixed orientation of the radical-pair forming molecules in the retina, the effect of the Earth's magnetic field would modulate the light signalling differently depending on the location of the molecule within the retina. This would allow for perceiving the magnetic field as visual modulation patterns (Ritz *et al.* 2000). Ritz *et al*. (2000) developed a model to illustrate how a field dependent radical pair process could affect processes involved in visual information transfer (Figure 6). For the purpose of illustration, the authors have chosen intensity as the modulated variable; other potential features that may be important are e.g. high contrasts in size or shape or a combination of several parameters. Since gradually changing intensity or colour patterns are hard to detect for the visual system, movement of the image on the retina may be necessary to detect the virtual visual pattern resulting from modulations by the Earth's magnetic field. We recently found that night-migratory garden warblers use head scans to detect the reference direction provided by the magnetic field (Mouritsen *et al.* 2004b, Paper I). The head scanning behaviour is therefore very well suited for a vision-mediated reception mechanism, especially when we consider that graded visual patterns are much easier to detect when they move than when they remain stationary.

While the results of all behavioural experiments introduced earlier, are consistent with a vision-mediated radical-pair-based magnetic compass activated by a blue-green photopigment, they fall short of providing conclusive evidence for a chemical sensing mechanism underlying the avian magnetic compass. Oscillating magnetic fields in the low radio-frequency range (1-50 MHz) have been proposed as a diagnostic test for a potential involvement of a spin related reaction mechanism (Ritz *et al.* 2000; Cintolesi *et al.* 2003; Henbest *et al.* 2004). Such fields would compete with the effects of the Earth's magnetic field and should therefore alter the ability of a radical-pair-based chemical sensor to integrate reliable directional information provided by the geomagnetic field. On the other hand, such fields should not affect magnetic orientation mechanisms based on magnetite-mediated magnetoperception (see following chapter). Oscillating fields of the radio-frequency range would have to be considerably stronger than Earth's field strength to affect a magnetite-based reception mechanism. Magnetic orientation performance of European robins has recently been shown to be disrupted when they are exposed to weak (< 0.2 times geomagnetic field strength) radio-frequency fields (Ritz *et al.* 2004; Thalau *et al.* 2005). As expected from theoretical modelling (Ritz *et al.* 2000), the effect of the applied fields depended on the alignment between the Earth's magnetic field and the artificially applied oscillating field (Ritz *et al.* 2004): parallel orientation of both field sources did not alter orientation performance of the birds. The experimental results show a distinct signature of the applied radiofrequency (Ritz *et al.* 2004; Thalau *et al.* 2005). They are in line

with expectations from a radical-pair-based process and at the current stage cannot be explained with any other known mechanism. Whatever is affected by the treatment, a radical pair process must somehow be coupled to aspects of orientation behaviour. These results indicate a resonance effect on singlet–triplet transitions and a coupling of this to a biochemical transduction process leading to compass orientation performance in birds. Therefore, they provide the strongest, albeit indirect, evidence that the biophysical mechanism underlying the magnetic compass of birds must involve a radical pair mechanism.

Research strategy to address the key untested prerequisites for vision mediated magnetoreception

During my Ph.D. studies, I aimed to experimentally test several of the above listed theoretical assumptions required for vision-mediated magnetoperception by using a multidisciplinary approach. The key untested prerequisite (**prerequisite 1**) for all further experiments was to demonstrate the **existence of the candidate molecule**, cryptochrome, in the retina of migratory birds while they perform a magnetic orientation task at night. To achieve this, we initiated a collaborative study with the group of Prof. Reto Weiler, Oldenburg University, using an immunohistochemical approach. Sequence identification of the garden warbler molecule was performed on the mRNA level using total RNA extracts from garden warbler retina as template.

The sole existence of cryptochrome molecule *per se* does not proof it's involvement in the suggested perception mechanism. To be potentially relevant for magnetoperception, at least some cryptochrome containing retinal cells must be active at night when the birds perform magnetic orientation (**prerequisite 2**). To test the **neuronal activity pattern** of retinal cells, we used the markers c-Fos and ZENK (acronym for the gene known in other species as *zif-268, egr-1, NGF-IA* and *krox-2*) (Mello *et al.* 1992).

After we knew that the candidate molecule does exist in the retina of night-migratory birds, my focus shifted towards the biochemical and biophysical properties of the molecule itself. To study the **intrinsic characteristics** of the molecule and thus allowing me to address **prerequisites** number **4, 5, 6** & **9**, I needed to study the protein in isolation. Therefore, I used the baculovirus/*Sf9* (*Spodoptera frugiperda*) insect cell system to recombinantly express the species-specific proteins in large amounts and subsequently study its biophysical properties.

II. 2. Background: Cryptochrome – the candidate molecule

Ritz *et al.* 2000 suggested the cryptochromes, a class of blue-light photoreceptors, to be the most promising magneto-sensory candidate molecules, since cryptochromes are currently the only class of molecules known to be present in vertebrates that are likely to fulfil the physical and chemical characteristics required to act as a primary magnetic sensor (Ritz *et al.* 2000). Cryptochromes are blue-light and ultraviolet (UV-A) photoreceptors in plants. They share sequence similarity to photolyases, a class of flavoenzymes catalysing repair of UV-damaged DNA via electron transfer mechanisms. The first cryptochrome gene to be identified was *Arabidopsis* Cry1 (Ahmad & Cashmore 1993). The gene product was named Cry1 from 'cryptochrome' previously proposed for 'cryptic' blue light receptor (Gressel 1979). Cryptochromes have now been found in other plants (e.g. Cashmore 1997; Small *et al.* 1995; Imaizumi *et al.* 2000, 2002) and in various animal lineages, including insects (Todo *et* al. 1996; Emery *et al.* 1998; Egan *et al.* 1999; Rosato *et al.* 2001), fish (Kobayashi *et al.* 2000), amphibians (Zhu & Green 2001), birds (Bailey *et al.* 2002; Mouritsen *et al.* 2004a; Möller *et al.* 2004) and mammals (Hsu *et al*. 1996; Todo *et al.* 1996; Kobayashi *et al.* 1998; Griffin *et al*. 1999; Kume *et al.* 1999). Recently a new type of cryptochrome was also found in

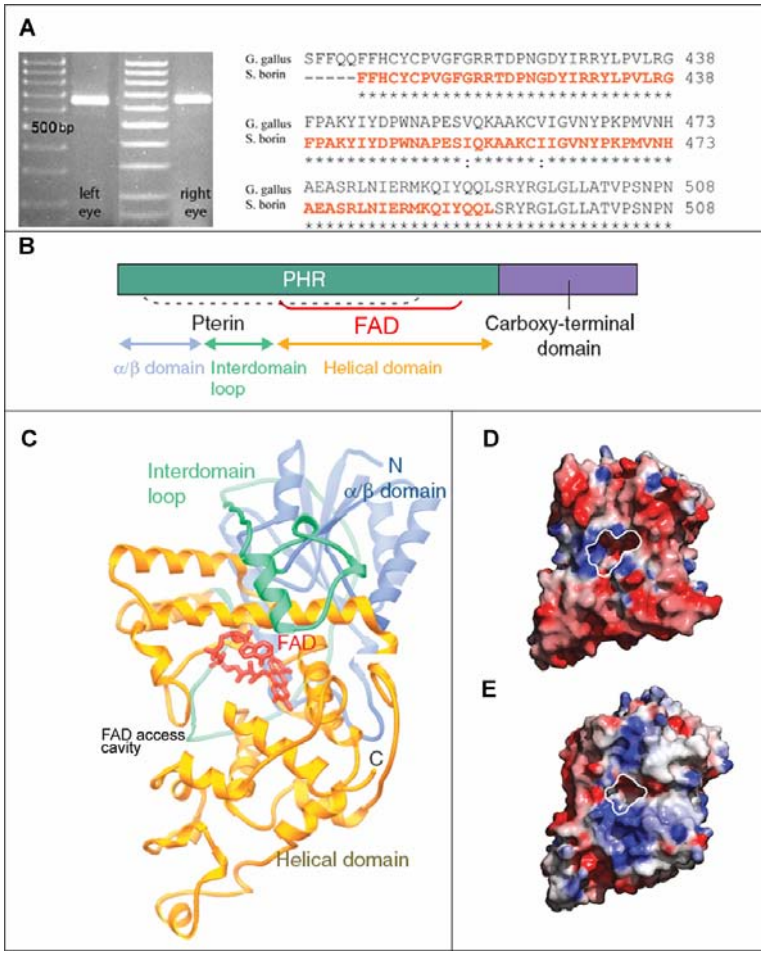

Figure 7: Cryptochrome, the candidate receptor molecule **A** Detection of a *gw*Cry1a transcript (expected fragment size 642 bp) in the retina of garden warbler (comparison between left and right eye). The alignment shows amino acid sequences of *gw*Cry1a and chicken Cry1; the FAD binding site is shown in red. **B** A schematic illustration of the representation of a typical photolyase/ cryptochrome superfamily protein: the N-terminal photolyase homology region (PHR) is highly conserved both between species and within members of the multigene family; it comprises binding sites for both cofactors and functions as the photoreceptor. The C-terminal extension differs in length and sequence between different molecules and is rather involved in specific signalling/binding interaction. **C** The overall folding of a Cry-DASH protein, *Synechosystis* sp.PCC6803 cryptochrome. **D**, **E** A comparison of the structures of the PHR regions of **D** *Arabidopsis* Cry1 and **E** *E. coli* DNA photolyase. White lines indicate the boundaries of the FAD access cavity; red and blue represent areas of negative and positive electrostatic potential, respectively (modified after Mouritsen *et al.* 2004a; Lin & Todo 2005).

cyanobacteria *Synechocystis* sp. (Hitomi *et al.* 2000) (Figure **7**C). This new type of cryptochrome was referred to as Cry-DASH, to indicate its relationship with cryptochromes found in *Drosophila, Arabidopsis, Synechocystis* and *Homo* (although Cry-DASH itself is not found in *Drosophila* or *Humans*).

Cryptochromes are implicated in multiple blue-light dependent signalling pathways in plants and animals (extensively reviewed in Cashmore *et al.* 1999; Deisenhofer 2000; Ahmad 2003; Bouly *et al.* 2003; Sancar 2000, 2003, 2004; Lin & Todo 2005). In animals, cryptochromes have been shown to play a role in circadian rhythms, either directly as components of the circadian pacemakers (Miyamoto & Sancar 1998; van der Horst *et al.* 1999; Sancar 2000, 2004) and/or as cellular signalling intermediates in the circadian clock of mouse (Shearman *et al.* 2000). In *Drosophila*, cryptochromes are more indirectly involved by feeding light information into the circadian clock (Busza *et al.* 2004; Stanewsky 2002). In plants, cryptochromes have been shown to be involved in de-etiolation responses such as inhibition of hypocotyls elongation (Ahmad & Cashmore 1993; Lin *et al.* 1995, 1998; Lin 2002) and anthocyanin accumulation (Ahmad *et al.* 1995) in leaf and cotyledon expansion (Cashmore *et al*. 1999; Lin 2002; Lin & Shalitin 2003), transitions to flowering (El-Din El-Assal *et al.* 2003), or regulation of blue-light regulated genes (Jiao *et al.* 2003). Cryptochromes are probably the evolutionary descendents of DNA photolyases, a class of flavoproteins that catalyse repair of UV-damaged DNA via light-dependent electron transfer reactions[1] (Carell *et al.* 2001). Photolyases and a plant cryptochrome of *A. thaliana* (Cry1) have recently been shown to form radical pairs upon photoexcitation (Giovani *et al.* 2003).

The defining characteristics of cryptochromes are N-terminal domains with marked similarity to DNA photolyases (Deisenhofer 2000; Brautigam *et al.* 2004) (Figure 7B). Within the amino-terminal photolyase homology region (PHR), cryptochromes and photolyases have similar three-dimensional structures, characterised by an α/β domain and a helical domain. Cryptochromes bind similar cofactors to photolyases but lack DNA repair activity (Malhotra *et al.* 1995; Lin *et al.* 1995) suggesting evolution of novel activities to explain the cryptochromes' role in signalling. Despite many sequence similarities and apparent functional analogy, plant and animal cryptochromes seem to have evolved independently from different ancestral photolyases, with animal cryptochromes more similar to type 6-4 photolyases and plant cryptochromes sharing greater sequence similarity type I microbial photolyases (Kanai *et al.*

[1] The photolyase–blue-light photoreceptor family is composed of cyclobutane pyrimidine dimer (CPD) photolyases and (6-4) photolyases CPD photolyases and (6-4) photolyases are involved in photoreactivation for CPD and (6-4) photoproducts, respectively (reviewed by Sancar 1996). CPD photolyases are classified into two subclasses, class I and II, based on amino acid sequence similarity.

1997). A further defining characteristic of both plant and animal cryptochromes are carboxy-terminal extensions of varying size, not present in photolyases. This extension, which is essential for a number of cryptochrome-specific functions, is longer in most plant cryptochromes than animal cryptochromes and generally less conserved than the PHR region. Cry-DASH proteins lack this domain (Lin & Shalitin 2003).

Crystal structures have been determined for three members of the photolyase/cryptochrome superfamily in various species: cyclobutane pyrimidine dimer (CPD) photolyase (*Escherichia coli*, *Synechococcus* sp., *Thermus thermophilus*) (Park *et al.* 1995), cry-DASH (*Synechocystis* sp.) (Brudler *et al.* 2003), and a plant cryptochrome (PHR domain of *Arabidopsis* Cry1) (Brautigam *et al.* 2004) (Figure 7C, D, E). The similarities of the three-dimensional structures of the crystallised PHR regions are remarkably high: all fold into two domains, an α/β domain and a helical domain, which are connected by a variable loop that wraps around the α/β domain (Figure 7B, C). The structure also includes a chromophore, flavin adenine dinucleotide (FAD). The FAD-access cavity of the helical domain is the catalytic site of photolyases, and it is predicted also to be important in the mechanism of Cryptochromes. FAD is deeply buried in the FAD-access cavity in all three proteins (Park *et al.* 1995; Brudler *et al.* 2003; Brautigam *et al.* 2004). FAD binds non-covalently to the protein in a U-shaped conformation with its adenine and isoallocazine rings positioned at the bottom of the cavity. Despite the overall similarity, the Cry1-PHR region has several structural characteristics that differ from photolyase and cry-DASH. In contrast to photolyases, the Cry1-PHR region does not have a generally positively charged DNA-binding groove, possibly indicating that cryptochrome may not bind DNA directly. In addition the surface of Cry1-PHR is predominantly negatively charged, except for a small concentration of positive charges near the FAD-cavity (Brautigam *et al.* 2004).

The catalytic mechanism of cryptochromes has not yet been fully elucidated, but some clues can be found in the mechanisms of CPD photolyases, where FAD plays the main catalytic role (Park *et al.* 1995) (mechanism summarised in Lin & Todo 2005). The exact role of FAD and the FAD-access cavity in the function of cryptochromes remains unclear, but it is conceivable that it may also be involved in electron-transfer reactions (Giovani *et al.* 2003).

These functional and structural characteristics make the cryptochromes suitable for a putative radical-pair-based magnetoreception system.

II. 3. Own contribution: *In vitro* evidence – shedding light on the candidate receptor molecule

II. 3.1. Results I: Identification and cloning of garden warbler cryptochromes

(Mouritsen *et al.* 2004a, Paper II ; Liedvogel *et al.* 2006a, Paper III)

Explanation and deeper understanding of the previously obtained behavioural results supporting an involvement of a light-induced radical-pair magnetoreception mechanism inevitably require the presence of a radical pair forming photopigment in the eye of the migratory bird (**prerequisite 1**).

Therefore, the experimental demonstration of the presence of cryptochrome in the retina of night-migratory songbirds was the key prerequisite for all further experiments along this line. During my Ph.D. studies, I managed to identify four members of the cryptochrome multigene family in the garden warbler (*gw*) (for exact cloning strategies see *Supplementary material B*). I obtained, cloned, sequenced and analysed full length sequences of *gw*Cry1a (GenBank accession no. AJ632120) and *gw*Cry1b (GenBank DQ838738) (Cry1a and 1b are alternative splicing products, Möller *et al.* 2004), as well as fragments of *gw*Cry2 (777 bp, GenBank AY739908) and *gw*Cry4 (237 bp) (Mouritsen *et al*. 2004a, Paper II; Liedvogel *et al.* 2006a, Paper III; *Supplementary material A & B*). For the European robin (*e*), an second night-migratory songbird, three members of the cryptochrome family (*e*Cry1a, 1b; *e*Cry2) have been identified and partially sequenced by Möller and colleagues (Möller *et al.* 2004).

An alignment of Cry1a/b from garden warbler and European robin with photolyase sequences shows that the three tryptophane residues (Trp382, Trp359 and Trp306 in *Escherichia coli* photolyase) involved in the electron transfer (Giovani *et al.* 2003) are also conserved in garden warbler and European robin cryptochromes (Martin Byrdin & Klaus Brettel, personal communication). Alignment and comparison of garden warbler Cry1a with the amino acid sequence and crystal structural analysis of *Arabidopsis* Cry1-PHR (Brautigam *et al.* 2004) reveals the following: *Aves* cryptochromes lack the flexible *Arabidopsis* N-terminal (aa 1-12; amino acid numbers refer to *Arabidopsis* Cry1-PHR residues, Brautigam *et al.* 2004) and garden warbler Cry1a directly starts with a section of relatively high homology at the structurally defined α/β domain (aa 13-139; 43 % identity on the protein level between garden warbler and *Arabidopsis*, 98 % identity between garden warbler and chicken; all analyses are based on WU-BLAST2 algorithms unless stated otherwise). As expected, the so called

"connector-domain", a segment connecting both structural domains, is less conserved and varies in length between *Aves* (73 aa) and *Arabidopsis* (77-aa between aa 140-216) (37 % identity between garden warbler and *Arabidopsis*, 98 % between garden warbler and chicken). Within the α domain, in which the flavin cofactor is bound (aa 217-495), the homology between garden warbler and *Arabidopsis* Cry1-PHR sequence show high homology again (59 % identity between garden warbler and *Arabidopsis*, 99 % identity between garden warbler and chicken). The high homologies between *Aves* and *Arabidopsis* in the α/β and α domain regions and the conservation of functionally important residues confirm that the N-terminal section of *Aves* Cry1 will be similar in domain organisation, structure and cofactor binding to *Arabidopsis* Cry1. As expected, C-terminal extensions differ highly between garden warbler Cry1a and *Arabidopsis* Cry1-PHR and plant and animal cryptochromes in general. The C-terminal domain generally is very flexible with remarkable structural and functional differences (different cryptochrome C-termini bind to different sets of interacting proteins) both within different members of the multigene family and between species. Based on theses considerations, I have chosen aa 1-509 of the garden warbler Cry1a sequence for the gwCry1-PHR-construct (see *Supplementary material A & B*; Liedvogel *et al.* 2006a, Paper III).

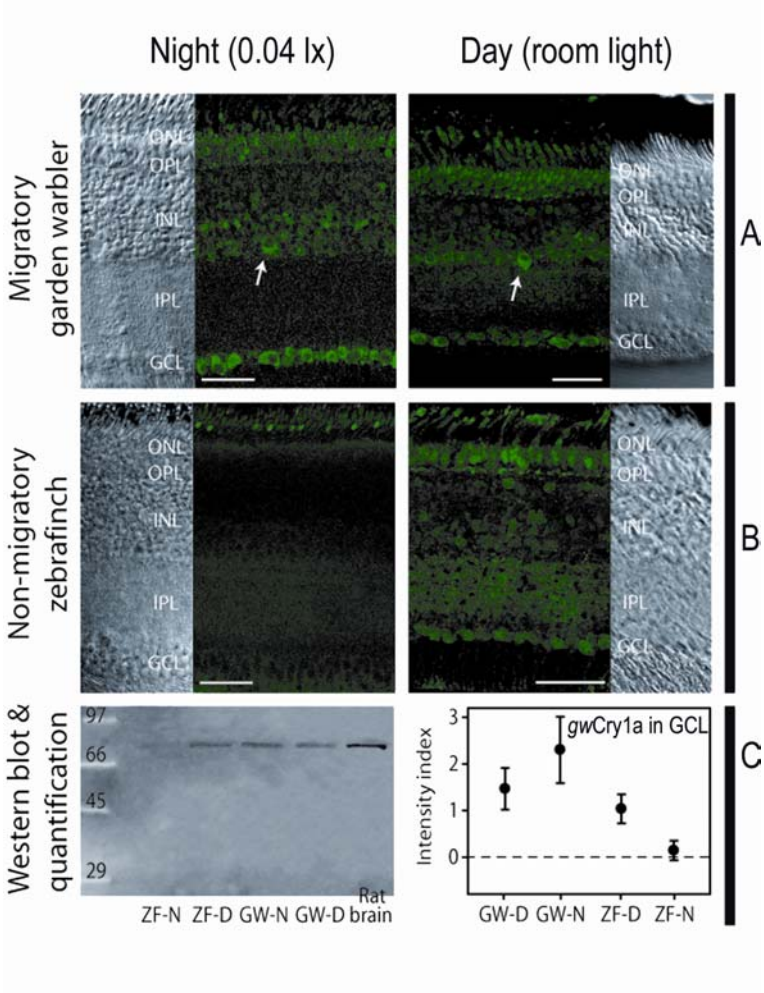

Figure 8: Comparison of Cry1(a) protein expression in retinae of migratory garden warblers and non-migratory zebra finches **A** Immunohistochemical staining of *gw*Cry1a protein during nocturnal magnetic orientation (*left*) and during the day (*right*) in migratory garden warblers. Large displaced ganglion cells marked with arrow. **B** Immunohistochemical staining of Cry1 protein during the night (*left*) and day (*right*) in non-migratory zebra finches. **C** Example of Western blot analysis confirming specificity of the antibody (*left*) and quantification of Cry1 expression in the ganglion cell layer (*right*). Dashed line indicates unspecific background level of expression. Scale bar = 40 µm; GCL = ganglion cell layer; IPL = inner plexiform layer; ONL = outer nuclear layer; OPL = outer plexiform layer (from Mouritsen *et al.* 2004a, Paper II).

II. 3.2. Results II: Expression pattern of cryptochrome in garden warbler retina

(Mouritsen *et al.* 2004a, Paper II)

By means of immunohistochemistry, we mapped the expression pattern of Cry1a in the retina of garden warblers performing magnetic orientation behaviour at night (Mouritsen *et al.* 2004a, Paper II). We could show that *gw*Cry1a[2] was predominantly found to be expressed in the cytosol of ganglion cells, displaced ganglion cells and in the photoreceptor layer (Figures 8 & 9), whereas *gw*Cry2 shows nuclear localisation (Mouritsen *et al*. 2004a, Paper II).

High cytosolic concentration of *gw*Cry1a in ganglion and displaced ganglion cells are favourable for a putative role of the cryptochromes as magnetic compass detectors rather than a control element of the internal circadian clock, since the cryptochromes would have to get to the nucleus in order to perform a regulatory clock function. Furthermore we found striking differences in cryptochrome expression levels between migratory and non-migratory birds at night (Mouritsen *et al.* 2004a, Paper II). Cry1 expression levels in non-migratory control species tested during the night (when awake) decrease to background (Mouritsen *et al.* 2004a,

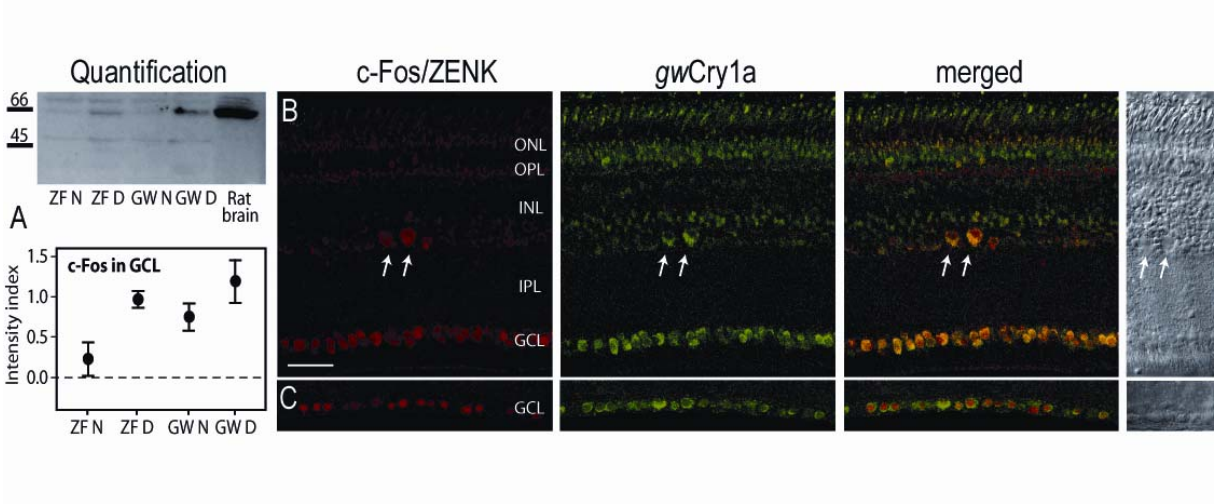

Figure 9: Colocalisation of Cry1a and neuronal-activity markers in a magnetically orienting garden warbler **A** Example of Western blot analysis confirming specificity of the antibody and quantification of c-Fos expression in the ganglion cell layer; immunosignal intensity was quantified relative to background intensity. Dashed line indicates unspecific background level of expression. **B**, **C** Immunohistochemical double labelling of *gw*Cry1a (cytosolic) with the neuronal-activity markers c-Fos (**B** cytosolic and nucleic) and ZENK (**C** exclusively nucleic; only the ganglion cell layer shown) reveal that *gw*Cry1a is found in night-active ganglion cells and in large displaced ganglion cells of the INL (arrows). Scale bar = 40 µm; for abbreviations see Figure 8 (from Mouritsen *et al.* 2004a, Paper II).

[2] Immunohistochemical staining data in Mouritsen *et al.* 2004a (Paper II), refer to Cry1 protein. At the state of publication, the presence of two alternative splicing products Cry1a (referred to as Cry1 in the publication) and Cry1b was not confirmed. Staining was performed with commercially available antibody directed against the C-terminal end of Cry1. Subsequent analyses (Liedvogel *et al.* 2006a, Paper III) reveal its specificity for the C-terminal part of garden warbler Cry1a (specificity of the antibody was confirmed by Western blot analysis probing the garden warbler specific Cry1a protein; Cry1b is not detected by the antibody used in Mouritsen *et al.* 2004a). To be concise, I will refer to *gw*Cry1a (Cry1 in Mouritsen *et al.* 2004a) whenever the immunohistochemical data refer to garden warbler specific staining in the following. For cryptochrome staining on zebra finch control tissue, I will keep the labelling, used in the paper (Cry1) as so far no alternative splicing products have been identified for this species.

Paper II; Haque *et al.* 2002) whereas *gw*Cry1a expression levels in migratory garden warblers remain high during the night (Figure 8). The circadian expression pattern found in our non-migratory control birds is consistent with data from the literature e.g. for domestic chicken (Fu *et al.* 2002; Haque *et al.* 2002). During the day, Cry1/*gw*Cry1a is found both in migratory and non-migratory species (Mouritsen *et al.* 2004a, Paper II). Differences in the Cry1/*gw*Cry1a expression pattern were particularly prominently found in displaced ganglion cells. In garden warbler retinae, displaced ganglion cells always show high expression levels, both during the day and during the night, whereas in zebra finches (*Taeniopygia guttata*) this particular cell type never showed Cry1 expression (Mouritsen *et al.* 2004a, Paper II) (Figure 8). *gw*Cry1a expression levels in migratory garden warblers are equally high, both during the day and during the night (Figure 8). This finding is further in line with the suggestion that a cryptochrome-mediated magnetic compass may also work during the day, where wavelength-dependent magnetic compass orientation has been observed (Wiltschko & Wiltschko 1995, 2002).

As previously mentioned, the cryptochrome-containing retinal cells must be active at night when the birds perform magnetic orientation in order to be potentially relevant for magnetoperception (**prerequisite 2**). We tested the neuronal activity pattern of retinal cells by using the neuronal activity markers c-Fos and ZENK (immediate early genes; for a further characterisation of these gene class, see section *III. 2* about *behavioural molecular mapping*), which are known to show vision-dependent expression in the retina of many animals (Dragunow & Faull 1989; Yoshida *et al.* 1993; Araki *et al.*1998; Fischer *et al.*1999) including chicken (Fischer *et al.*1999), and their expression requires neuronal activity (Worley *et al.* 1991; Chaudhuri *et al.* 1995). In our experiment on garden warblers performing magnetic orientation behaviour at night, both activity marker genes colocalised with Cry1a in all ganglion cells and large displaced ganglion cells, which are the cell types sending visual information from the eye to the brain (Figure 9). This diagnostic tool of visualising the cells' activity status can not be applied for retinal photoreceptor cells as they do not communicate via axon potentials. From the cell-anatomical point of view however, membrane structures of the photoreceptor cells would provide the ideal prerequisite for a fixed and highly oriented ensemble of molecules, necessary for a radical-pair-based magnetoperception mechanism. Electrophysiological recordings in retinal ganglion cells are necessary to provide a final proof of whether the cryptochromes are involved in the process of magnetoreception or not.

II. 3.3. Results III: Recombinant expression and biophysical characterisation of garden warbler cryptochromes

(Liedvogel *et al.* 2006a, Paper III)

In order to further investigate, whether cryptochromes could be primary magnetic compass detectors, we must characterise their molecular properties, i.e. it is essential to study the biophysical properties of cryptochrome protein in isolation (**prerequisites 4**, **5**, **6** & **9**). Having the full length cDNA sequence of garden warbler cryptochrome candidates on hand, I recombinantly expressed *gw*Cry1a and *gw*Cry1b in a Baculovirus/*Sf9* insect cell system (Liedvogel *et al.* 2006a, Paper III), one of the most powerful and versatile eukaryotic expression systems available (Luckow & Summers 1988; Blissard & Rohrmann 1990).

To probe for potential effects of the C-terminal extension of both alternative splicing products on the reaction process or absorption spectra, I additionally expressed the Cry1 photolyase-homology domain (PHR), a highly conserved N-terminal domain of the superfamily of photolyases and cryptochromes, which is identical for *gw*Cry1a and *gw*Cry1b (the N-terminal sequence chosen for the *gw*Cry1-PHR-construct is based on considerations, discussed above). For details of the expression and purification procedures see *Supplementary material A*. I expressed *gw*Cry1a (628 aa; ~69 kDa), *gw*Cry1b (595 aa; ~65 kDa) and *gw*Cry1-PHR (510 aa; ~56 kDa) as soluble His-tagged fusion proteins and purified the garden warbler proteins to near homogeneity by means of immobilised metal affinity chromatography (IMAC). I managed to purify all three garden warbler cryptochromes with their flavin cofactor built in. This was the first successful expression of an animal cryptochrome as a holoprotein (meaning cryptochrome apoprotein plus flavin cofactor built in) in a heterologous eucaryotic expression system. I overcame the known difficulties (these problems were probably caused by missfolding of the C-terminal extension) in expression the animal cry-holoprotein, and thus achieved this project, which has been previously unsuccessfully aimed for in many other labs. In a novel and independently developed strategy, I added an extra on-column denaturation/refolding step to the purification protocol (for details on the purification protocol, see *Supplementary material A*).

Successful expression of the holoprotein was the crucial prerequisite for further biophysical characterisations of the molecule, and thus for testing its putative role in the process of light-mediated magnetoperception. The reason is that the suggested radical-pair process is based on the interaction of cry-apoprotein and the flavin cofactor.

Once expressed and purified, we were able to experimentally investigate several key properties of the migratory bird cryptochromes, assumed in the model calculation by Ritz and colleagues (2000). We could show that cryptochromes of migratory garden warblers are exited by light (**prerequisite 4**) and that their absorption spectrum has a range that corresponds to a wavelength range known to elicit magnetic orientation performance in behavioural experiments (**prerequisite 5**). Furthermore, we could experimentally validate the crucial model-assumption (**prerequisite 6**) that garden warbler cryptochromes with flavin as a cofactor form a radical pair intermediate and that this intermediate state is very long lived (~ 10 ms), so that it can be differentially affected depending on the direction of Earth's strength magnetic fields (Liedvogel *et al.* 2006a, Paper III) (Figure 10).

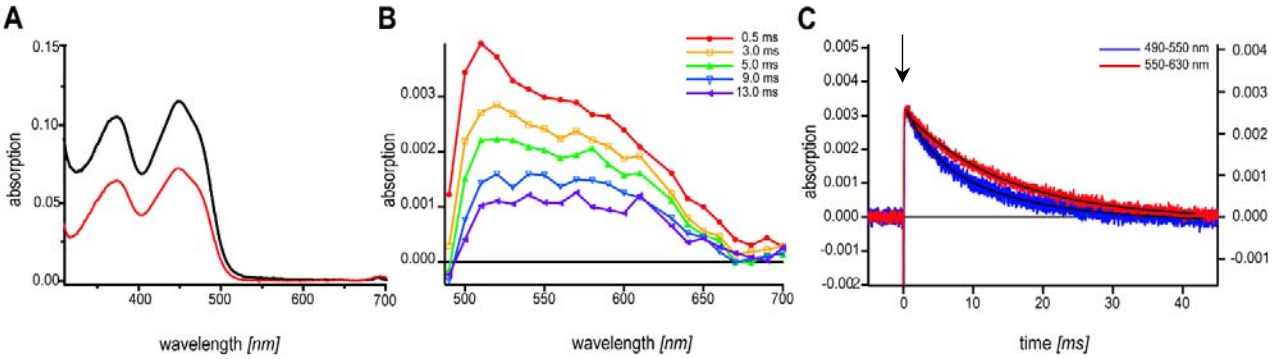

Figure 10: Biophysical characterisation of Cryptochrome from migratory garden warblers. A UV/vis absorption spectra for recombinantly expressed garden warbler cryptochrome protein (Cry1a and Cry1-PHR). Both *gw*Cry1a (red line) and *gw*Cry1-PHR (black line) show absorption maxima at 450 and 366 nm. **B** Transient absorption spectra obtained for *gw*Cry1a as a function of wavelength (monitoring beam). The five graphs reflect the optical properties of the sample at different delay times t_{delay}, between laser flash (at time t = 0) and monitoring window (t_{delay} = 0.5, 3, 5, 9, 13 ms; width of monitoring window is 500 µs). Comparison of the five spectra shows that the pronounced absorption peak at early life times around 510 nm (most prominent in the red curve) has a much shorter life-time than the features, observed at higher wavelengths. **C** Transient absorption time profiles for *gw*Cry1a of the migratory garden warbler. The absorption of the sample is detected as a function of time after the laser flash for two wavelength regions (red curve: 490-550 nm, left scale; blue curve, right scale: 550-630 nm). The decay shown in the red graph can be modelled as a double exponential with half-lives (meaning the signal drops to half of its original value at t = 0) of 3 ms and 10 ms, respectively whilst the longer-lived blue decay was modelled with a best fit half life of 10ms. These calculations show that Cry1a radicals from migratory garden warbler are long-lifed (in the millisecond range). Because the sensitivity of a radical-pair system to weak magnetic fields increases significantly with longer life times of the radical species (Ritz *et al.* 2000), our results further strengthen the suggested role of cryptochromes as primary magnetoreceptor molecules (modified after Liedvogel *et al.* 2006a, Paper III).

III. **Magnetic orientation in birds** – transduction and processing

III. 1. **Reviewing the literature:** What to conclude from neuroanatomical studies and electrophysiological recordings?

As mentioned earlier (see section *II. 1.1. Magnetite*), studies focussing on the physiology of magnetoperception revealed iron deposits located in the head region of birds. Particularly iron structures located in the upper beak of pigeons are discussed as one possible magnetoreceptor (Walcott *et al.* 1979; Presti & Pettigrew 1980; Beason & Nichols 1984; Beason & Brennan 1986; Fleissner *et al.* 2003). The ophthalmic branch of the trigeminal nerve is located close to the demonstrated iron structures, and therefore could potentially form a suitable transduction pathway for neuronal processing of magnetic information. Motivated by this consideration, electrophysiological recordings were performed on the ophthalmic branch of the trigeminal nerve structure with the goal to record nerve responses to magnetic stimuli. Experiments conducted by Beason & Semm claimed to find positive responses to magnetic stimuli in both the ophthalmic nerve and the trigeminal ganglion (Beason & Semm 1987, 1996; Semm & Beason 1990). As outlined above, these studies have been proven difficult to replicate and caution is advised in interpretation any conclusions drawn from these findings. However, recently conducted magnetic anomaly discrimination experiments by Mora and colleagues support the idea that the ophthalmic nerve is involved in magnetic sensing, probably in relation to a magnetic map or signpost sense (Mora *et al.* 2004).

In addition to studies focussing on the peripheral nerveous system, magnetosensitive cells were also reported in distinct brain areas. Electrophysiological recordings from the optic tectum and the pineal of the pigeon (Semm *et al.* 1984; Semm & Demaine 1986) and bobolink (*Dolichonyx oryzivorus*) (Beason & Semm 1987) indicated the presence of neurons that respond to changes in the direction of the ambient magnetic field in the presence of light. The study does not include analysis of potential wavelength dependencies, only the presence or absence of light was evaluated. These findings are in line with the suggestion of a light-dependent magnetic receptor associated with the visual system but, again, these findings have also been difficult to replicate. Another brain area known to be involved in spatial memory, e.g. caching and retrieving food items, and therefore of potential interest for the field of orientation and navigation, is the hippocampal formation (Sherry & Vaccarino 1989; Hampton & Shettleworth 1996; Smulders & deVoogd 2000). Recent studies suggest an involvement of the hippocampal formation in the map but not the compass sense (Bingman *et al*. 1999;

Gagliardo *et al.* 2001). All studies trying to identify brain areas involved in the process of magnetoperception have so far provided vague hints along this line, but distinct key areas responsible for processing orientation-relevant compass-information still remain to be identified.

III. 2. **Own contribution:** Linking migratory orientation behaviour to its underlying neuronal circuits

The global phenomenon of bird orientation comprises different components which have to be integrated in order to fully understand the interplay between primary detection, neuronal integration and resulting behaviour. In the previous chapter, I have focussed on the primary detector level and mentioned that current evidence suggests that visual sensing of magnetic compass information occurs through the eyes by means of a light-activated, radical-pair-based magnetodetector (Ritz *et al.* 2000, 2004; Wiltschko & Wiltschko 2002; Muheim *et al*. 2002; Wiltschko *et al.* 2003a; Mouritsen *et al.* 2004a, Paper II). No matter where the primary magnetic compass sensors are located, the detected information provided by the magnetic field has to be integrated and sent to higher integration centres in the brain for further processing.

Behavioural molecular mapping – a new methodological approach to link behaviour to neuronal processes in the brain

When I started my Ph.D. studies, we faced the problem that no brain areas relevant for compass-orientation had been identified. Therefore, we decided to use *behavioural molecular mapping*. Behavioural molecular mapping allows researchers to analyse the entire brain and thereby identify brain areas, which are involved in integrating and processing information relevant for any given behaviour. Behavioural molecular mapping has been developed for studies focussing on the song-system of songbirds and has not been previously applied in the field of bird orientation (except one study in pigeons, which identified portions of the hippocampal formation and the medial striatum as possible sites of plasticity associated with homing; Shimizu *et al*. 2004). When an animal performs a particular behaviour, most forebrain areas[3] activated during this behaviour will increase the expression of neuronal activity

[3] It is important to notice that in a given brain region, different proportions of cells may be recruited by different stimuli (Mello *et al.* 1992) and it must not be inferred that all neurons respond with the same complement of IEGs. Potential neuronal activity evoked by magnetic orientation will not be detected in some areas with our method. Areas that do not show activity dependent ZENK induction are primary thalamic recipient neurons of the cerebrum (field L2, entopallium, and nucleus basorostralis), the globus pallidus, and parts of the thalamus (Mello & Clayton 1995; Jarvis 2004) e.g. the nucleus of the basal optic root (nBOR), where magnetosensitive neurons have been reported (Semm *et al.* 1984; Semm & Demaine 1986).

dependent *immediate early genes* (IEGs) in the brain, approximately in the proportion to the amount of neuronal activity in the given brain area (Jarvis & Nottebohm 1997). We used two different IEGs as neuronal activity markers in our study, namely ZENK (acronym for the gene known in other species as *zif-268, egr-1, NGF-IA* and *krox-2*) (Mello *et al.* 1992) and c-Fos (e.g. Clayton 2000). mRNA expression of the activity dependent marker genes can be detected from about 10 minutes after occurrence of the stimulus inducing neuronal activity. The temporal course of ZENK expression is well studied: it peaks after approximately 30 minutes of neuronal activation, and remains detectable for about 45-60 minutes (Jarvis & Nottebohm 1997).

These outlined characteristics of the technique make it ideally suited for the experiments I carried out during my Ph.D. studies, which aimed to directly link magnetic orientation behaviour during migration (the target behaviour) to brain area(s) processing information during this specific behavioural task.

Experimental paradigm

In our experiments, we mapped the IEG mRNA expression patterns by means of radioactive *in situ* hybridisation. The radioactive signal mirrors the brain areas that were active during at least some periods within the last 60 minutes of the bird's life.

As already noted, the technique of behavioural molecular mapping requires a carefully defined and controlled behavioural paradigm. Carefully designed control conditions are necessary to separate between the different components of any given behaviour. Our target behavioural repertoire, "magnetic orientation in night-migratory songbirds", consists of **motor**-components (wing flapping and jumping around on the perch during periods of migratory restlessness) as well as the **sensory** component required for magnetic sensing.

In an attempt to isolate the specific sensory component of magnetic-field perception, we tested birds under various magnetic scenarios as well as different wavelength conditions, which have been shown to alter orientation performance in behavioural experiments (e.g. Wiltschko *et al.* 1993; Mouritsen 1998a). To control for the effect of movement, we tested birds sitting still but awake and birds showing migratory restlessness in an otherwise identical orientation arena (light levels and magnetic conditions were kept constant).

As the target behaviour of magnetically directed migratory restlessness only occurs during night time, an additional **circadian** or **light-dependent** component might be reflected in the observed brain activity patterns. To control for a putative effect of the time of day and/or light condition, we also tested birds during the day under full room light and at different times of the night.

To effectively correlate the activity patterns in the brain with the behaviour of the animals, behavioural molecular mapping also requires careful observation of the behavioural repertoire the bird performs during the last one hour test before the bird is sacrificed. To keep gene expression noise due to variable motor behaviour low and to allow for real time observation of the birds behavioural repertoire during the entire testing period, we designed a novel orientation cage variant consisting of a cylindrical transparent Plexiglas cage fitted with a circular perch (see Figure 1D). Infrared cameras mounted to the top and to the side of the cage allowed us to observe and later to quantify all behavioural components of the test birds at day and night without disturbing the animal (Figure 1E, F). In this new orientation cage, our birds showed very stereotype migratory restlessness behaviour by performing wing whirring while perched, or they were sitting quietly on the perch. The birds' behaviour in our cages is very consistent and thus easier to separate the behavioural components, than it would have been in Emlen funnels, where the birds tend to show rather unspecific jumps and flights towards the walls and sliding back to the bottom of the funnel.

III. 2.1. Results I: Migratory birds use head scans to detect the magnetic field

(Mouritsen *et al.* 2004b, Paper I)

The first step and fundamental basis for all behavioural molecular mapping experiments I carried out during my Ph.D. studies was careful observation of the behavioural components the bird performs during the process of magnetic orientation. When observing the bird's behaviour in the new orientation cage during migratory restlessness, we noticed a very stereotype behaviour besides the well-known migratory restlessness behaviour. The birds turned their head to one side and back to the longitudinal body axis, often followed by a turn towards the opposite direction; this characteristic behaviour was termed "head scanning". One turn of the bird's head from the body axis position to an angle clearly more than 60° to the left or right, followed by the subsequent return of its head to the straight-ahead position while the bird remained at the same spot, was defined as one "head scan" (Mouritsen *et al*. 2004b, Paper I). The birds almost tripled their head scan frequency when they were exposed to a zero magnetic

field with no magnetic information available at all, compared to a natural magnetic field. Furthermore, the frequency of head scans increased significantly during the period just before onset of migratory restlessness behaviour (Mouritsen *et al*. 2004b, Paper I). We could also show, that the very first directional choice after a head scan in a natural magnetic field leads to an above-chance level of correct directional choices. This effect is not seen in a zero magnetic field. These findings led to the conclusion that night-migratory birds seem to use head scans to detect the reference direction provided by the ambient magnetic field. The use of head scans is a further indication that the magnetoreceptor is located in the bird's head, which is in line with a light-based magnetoreceptor in the birds' eyes.

III. 2.2. Results II: Cluster N – Identification of a brain area involved in night vision and possibly magnetic compass -sensing

(Mouritsen *et al.* 2005, Paper IV)

By using behavioural molecular mapping, we discovered that at least two species of night-migratory songbirds (garden warblers and European robins) possess a tight cluster of brain regions (Cluster N; "N" for night-time activation) highly active only during night time vision (Figure 11). Cluster N is located at the dorsal surface of the forebrain and is part of or adjacent to a known visual brain region, the "visual Wulst" (Wulst meaning bulge) (Mouritsen *et al.* 2005, Paper IV). In contrast to the strong night-time activation, Cluster N showed no significant ZENK induction during the day. Furthermore, in non-migratory controls (canaries, *Serinus canaria*, and zebra finches), this cluster of regions did neither show increased brain activity when tested during the day nor during the night when awake (Mouritsen *et al.* 2005, Paper IV). These findings led to the conclusion that Cluster N seems to be specific to night-migratory birds during night-time (low light level conditions). Given the neuroanatomical localisation of Cluster N and its likely relationship to the visual Wulst, we wanted to test, if neuronal activation in Cluster N requires visual input. To test this hypothesis, we tested night-migratory birds under the same scenario but with both eyes covered by light tight eye cups. This treatment prevented any light stimulus from reaching the eye. Blocking visual input resulted in strong reduction of neuronal activation in Cluster N. Our finding suggests an involvement of Cluster N in visual processing during night time, what would be in line with the predicted visual nature of magnetic and star compass orientation. By direct comparison of ZENK expression patterns from birds having both eyes covered and untreated controls, which had been collected at similar time points, we could further show that the increased activity in Cluster N we found in night-migrating birds is not due to circadian effects.

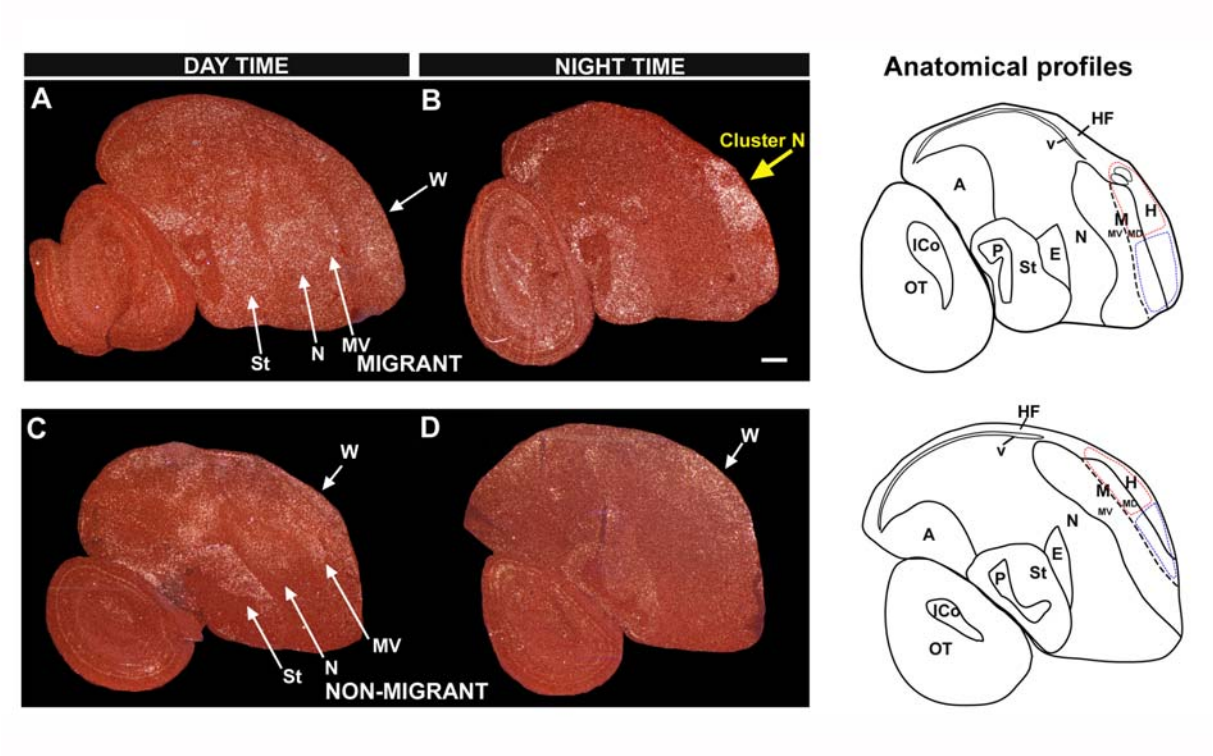

Figure 11: Neuronal activation patterns in both migratory and non-migratory birds at night time and during the day Darkfield pictures of parasagittal sections of a night-migratory garden warbler (**A** & **B**), and a non-migratory zebra finch (**C** & **D**); anatomical profiles are shown to the right (the red and blue lines encircle the areas used for quantification). **A** and **C** show the neuronal activation pattern during the day, **B** and **D** mirror the activation pattern at night time. (Dorsal is up, rostral is right. White silver grains show ZENK expression, visualising neuronal activation; the red colour of the brain tissue is due to Nissl staining. Scale bar = 0.5 mm; A = arcopallium; DNH = dorsal nucleus of the hyperpallium; E = entopallium; H = hyperpallium; HF = hippocampal formation; ICo = inferior colliculus; IHA = intercalated hyperpallium apicale (HA); M = mesopallium with ventral (MV) and dorsal (MD) part; N = nidopallium; OT = optic tectum; P = pallidum; St = striatum; v = ventricle; W = visual Wulst (from Mouritsen *et al.* 2005, Paper IV).

Thus, we suggest that, in night-migratory songbirds, Cluster N is involved in some kind of enhanced night vision, and that it could be integrating vision-mediated magnetic and/or star compass information for night time navigation.

III. 2.3. Results III: Lateralised activity patterns in Cluster N in a migratory bird

(Liedvogel *et al.* 2006b, Paper V)

In a series of subsequent experiments, I aimed to further characterise the functionality of Cluster N by performing a more detailed analysis of the brain area with respect to magnetic compass orientation. As behavioural experiments have suggested a strong asymmetry in magnetic compass orientation (Wiltschko *et al.* 2002a) as well as in general visual tasks (e.g. Rogers & Deng 1999), I examined the effect of lateralisation on Cluster N visual activity in European robins at night, and found a significant dominance of the right hemisphere

(Liedvogel *et al.* 2006b, Paper V). Furthermore, we analysed Cluster N expression in garden warblers under various magnetic scenarios as well as different light/wavelength conditions. If Cluster N – as a possibly higher integration area – is integrating magnetic field information, one could assume this area to show distinctly different activation pattern depending on the magnetic field the birds were exposed to. But when interpreting the results, we have to keep in mind that the assumed underlying magnetodetection mechanism is light-based with the light itself being the primary signal. Hence, whenever light of relevant wavelengths is available – as given in the experiments presented here – radical-pairs should be formed upon photoexcitation. Although the singlet-triplet-interconversion is sensitive to the magnetic field, leading to putative signal modulations, the signal due to photoexcitation itself will be present in any case, even in a true-zero magnetic field and it will have to be processed in the brain. The method of *behavioural molecular mapping* only visualises average overall neuronal activation in a certain brain area, and signal modulations due to the magnetic field would only be detectable if it strongly effects the overall activation pattern in a given brain area and if this effect is highly consistent over a 1 hour time period.

We did not find any significant changes in expression level of neuronal activity in Cluster N under different magnetic conditions. This suggests that the method of behavioural molecular mapping might be too insensitive to detect the magnetic field modulation pattern of the primary visual signal. An alternative explanation would be that the magnetic field, for example, up-regulates activity in some neurons while it down-regulates the activity of others. As our method only allows visualising the overall activity level of the brain area in focus, small modulations would not be detectable. To measure potential direct responses of neurons in Cluster N to changes in the ambient magnetic field, electrophysiological recordings are necessary. I performed an electrophysiological pilot study in collaboration with Prof. Georg M. Klump and Henrik Mouritsen. The electrophysiological recordings data from neurons of Cluster N obtained during this pilot experiment did not, however, reveal any correlation with the magnetic stimuli we applied.

We also compared Cluster N activity patterns between garden warblers exposed to red light (45 minutes exposure to red light; peak at 650 nm), which disturbed orientation ability in behavioural experiments (e.g. Wiltschko *et al.* 1993) and control birds exposed to white light. We found no significant effect of the experienced wavelength on Cluster N activity levels. Wiltschko *et al*. (2004b) found that robins were able to re-establish their orientation ability under red light after being pre-exposed to the monochromatic red light condition for one hour.

The exact characteristics of this adaptation process as well as details about the time period of pre-exposure, which is necessary to re-establish the orientation ability, is not precisely known at the current stage and our data may only be cautiously interpreted at the current time. It is possible, that light-mediated signals still reach Cluster N, but the magnetic modulations are either absent or so unusual that the bird needs time to interpret them correctly. However, as long as red light signals reach Cluster N, it will need to process the information and thus be active.

III. 2.4. Results IV: Movement-driven gene expression in migratory songbirds

(Feenders *et al.* 2006, Paper VI)

Comparison of the activity patterns between birds sitting still and birds showing migratory restlessness revealed striking differences. We found a distinct and highly consistent movement-related activity pattern in the birds' brain, independent of time of day (Feenders *et al.* 2006, Paper VI). Brain activity levels of sitting birds were generally very low – except for activity in Cluster N at night-time and in day-vision areas in birds tested during the day (Mouritsen *et al.* 2005, Paper IV). Detailed analyses of the movement-induced activity pattern revealed that the motor-dependent activity pattern mirrors an inverse picture of the neuronal activation pattern known from vocal-learning bird species (songbirds, parrots, hummingbirds) during song learning and production. In our birds showing migratory restlessness, brain tissue neighbouring the well characterised (e.g. Jarvis *et al.* 2000) seven song nuclei showed high activity, while the song nuclei themselves were not active. In comparison, singing birds showed high neuronal activity levels in the song nuclei but only little expression in the brain areas surrounding the nuclei. Three additional forebrain areas and parts of the cerebellum could further be identified as movement-related brain regions. The movement-related activity pattern was similar in different songbird species showing different types of motor activity (garden warblers performed migratory restlessness, whereas zebra finches showed regular movement in the cage, e.g. hopping/ flights up and down the perch, running on the ground). Our findings therefore suggest that the eleven identified brain regions are general features of songbirds and maybe of birds in general. Interpreting our findings within an evolutionary context, our data provide evidence that the song system may have developed out of a pre-existing motor pathway, which might be organised parallel to the song-circuit. This interpretation seems plausible, as singing behaviour includes several motor-components, such as muscles control of the beak, the tongue, the syrinx and the lungs. Therefore using a pre-existing motor-pathway

for the development of a system specialised for learning and producing new vocalisations seems very plausible.

IV. Conclusion and future research

As reviewed in the previous sections, the orientation cues and compass systems used by migratory birds to orient on their fascinating journeys covering thousands of kilometres are quite well understood. In contrast, the molecular and cognitive mechanisms of physiological integration- and transduction pathways are still not fully elucidated. During my Ph.D. studies I focussed on achieving a better understanding of the interplay of behavioural, molecular and neurophysiological processes of the global phenomenon of magnetic compass orientation.

The results presented in this thesis provide new experimental support of a light-dependent magnetoreception mechanism in migratory birds – both on a molecular and a neurobiological level. During my Ph.D. studies, I identified four members of the cryptochrome candidate class of primary magnetoreceptor molecules and showed that they are expressed in garden warbler retina. I succeeded in recombinantly expressing and biophysically characterising garden warbler cryptochrome Cry1a and Cry1b as well as their highly conserved N-terminal photolyase homology domain. This was the first successful recombinant expression and purification of an animal cryptochrome as a holoprotein with its flavin cofactor built in, from a heterologous eukaryotic expression system. The results of my biophysical characterisation of the putative receptor molecule agree well with predictions from behavioural experiments and experimentally confirm several key untested prerequisites assumed in the model calculations of Ritz *et al.* (2000). We mapped the expression pattern of garden warbler Cry1a in the eye, the suggested primary sensory organ for a light-dependent magnetoperception process. We have shown that *gw*Cry1a is predominantly expressed in the cytosol of ganglion cells and large displaced ganglion cells, which show high levels of neuronal activity at night when the birds performed magnetic orientation. *gw*Cry1a was also found to be strongly expressed in photoreceptors.

On the behavioural level, we found a characteristic head scanning behaviour, which birds seem to use to detect the reference direction provided by the Earth's magnetic field. In the context of vision-mediated magnetic compass detection, this finding suggests that movement of the image on the retina may be needed in order to detect the magnetically modulated visual pattern, which

is in line with the predictions assumed from theoretical modelling. The fact that the visual system is much better at detecting patterns moving over the retina rather than stationary graded patterns further strengthens the argument that head scanning behaviour might support vision-mediated magnetoperception.

Using behavioural molecular mapping, we identified a brain cluster in the forebrain of two species of night-migratory birds being highly active during night time, when the birds were orienting magnetically. We suggest that Cluster N of night-migratory songbirds is involved in enhanced night vision, and that it could be integrating vision-mediated magnetic and/or star compass information for night time navigation. We found an asymmetry in visual processing in Cluster N with a significant dominance of the right hemisphere.

Despite much recent progress in the field of magnetic compass mechanisms of migratory birds, there are still many open questions (most of them are summarised in Mouritsen & Ritz 2005). For instance, it is essential that the motion of the radical-pair forming molecule is restricted; otherwise the molecule cannot function as a primary magnetodetector. Therefore, one of the remaining untested prerequisite for a light-mediated magnetoperception mechanism is the oriented nature and fixed position of the primary receptor molecule. In photoreceptor cells, the highly oriented membrane structures would provide a well suited substrate to allow for fixed orientations of cryptochromes. Other candidate anchor structures in ganglion and displaced ganglion cells are cytoskeleton proteins and cytosolicly embedded membranes. However, at the current stage we do not know how cryptochromes are spatially organised in any cell type. To prove this, further experiments determining the localisation of the protein on an ultramicroscopic scale are necessary.

Electrophysiological recordings from retinal ganglion cells could provide the final proof of eye-mediated magnetodetection, if their membrane potential responds to changes in orientation of Earth's strength magnetic fields.

So far, we do not anything about the signalling pathways by which potential primary magnetic effects on cryptochromes are affecting the cells' membrane potential. This question can be addressed and further extended by future studies, following up the methodological approach of recombinant protein expression, I established for gwCry1a, gwCry1b and gwCry1-PHR.

At the current stage, the primary magnetoreceptor molecule remains unknown. Any cryptochrome or combinations of several members of this multigene family are likely candidates to be involved in the primary perception process. Thus, ongoing molecular analysis of garden warbler cDNA is necessary to reveal all members of the multigene family. Having the full length sequence information of further candidates available allows for subsequent recombinant expression and further testing of their specific biophysical properties. Having an expanded set of candidate molecules (e.g. further including *gw*Cry2 and *gw*Cry4) in isolation on hand, this sets the scene for examining putative magnetic field effects on the biophysical characteristics of each of the candidate molecules.

Any signal encoding magnetic field information will be sent to the brain. During my Ph.D. studies, we identified Cluster N, a brain region predominantly processing visual information during night time. These findings further support the hypothesis that light-mediated magnetic compass signals originate in the retina and that they are sent to the brain as visual information. So far, we could not ultimately prove, whether Cluster N really is the brain area integrating and processing magnetic compass information. To test this, electrophysiological recordings in Cluster N should provide further insight into the neurophysiology of Cluster N with respect to magnetic stimulation. An alternative approach to test for the functionality of Cluster N in night-migratory birds would be lesion experiments. If Cluster N is involved in magnetic compass orientation, behavioural experiments could test whether magnetic orientation is disrupted in birds with their Cluster N lesioned. Another promising line of research should focus on day migrants. All studies carried out during my Ph.D. studies focused on night-migratory birds. At the current stage we do not know if light-mediated magnetoreception work for all bird species, including day migrants. Another question along this line and key prerequisite to answer the previous question is the following: Does light-mediated magnetoreception work at all times of day?

Despite the fact, that there are still many open questions before we fully understand the phenomenon of magnetic compass orientation in birds, we now stand at a promising starting point: we have sensory hypotheses, which are based on a theoretical framework and which are strongly supported by behavioural, molecular and neuroanatomical evidence. This knowledge provides ideal prerequisites to further elucidate the molecular and physiological processes underlying magnetic compass orientation in birds and should finally allow us to achieve an overall understanding of the cognitive processes of one of the most interesting and lasting challenges in sensory biology (Mouritsen & Ritz 2005; *Science* (2005), **309**).

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Abbreviations

α	anti
A	arcopallium
aa	amino acid
AcNPV	*Autographa californica* nuclear polyhedrosis virus
AMP	anterior motor pathway
Amp	ampicillin
A. thaliana/At	*Arabidopsis thaliana*
AVP	anterior vocal pathway
BLAST	basic logical alignment search tool
bp	base pair
BV	baculovirus
cDNA	complementary DNA
Chl	chloramphenicol
CPD	cyclobutane pyrimidine dimer
Cry	cryptochrome
Da	Dalton (mass of one hydrogen atom)
DASH	new type of cryptochrome found in cyanobacteria; DASH underscores its relationship to *Drosophila, Arabidopsis, Synechocystis* and *Homo*
DMSO	dimethylsulfoxide
DNH	dorsal nucleus of the hyperpallium
dNTP	deoxyribonucleotide triphosphate
E	entopallium
E. coli	*Escherichia coli*
f1 origin	f1 phage origin of DNA replication
FAD/FADox	flavin adenine dinucleotide (fully oxidised form)
FADH•	flavin adenine dinucleotide (semiquinone radical)
$FADH_2/FADH^-$	flavin adenine dinucleotide (fully reduced form)
FASTA	Fast-all (fast protein or nucleotide comparison)
Field L (1, 2, 3)	field L (1,2,3)
GCL	ganglion cell layer
H	hyperpallium
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HF/Hp	hippocampal formation/hippocampus
$(His)_6$-tag	hexahistidine affinity tag
ICo	intercollicular complex
IEG	immediate early gene
IHA	interstitial region of the hyperpallium apicale (HA)
IMAC	immobilised metal affinity chromatography
IPL	inner plexiform layer
K	Kelvin
Kan	kanamycin
kb	kilobase
kDa	kilodalton
lacZ alpha	beta galactosidase gene alpha fragment

M	mesopallium
MCS	multiple cloning site
MD	dorsal mesopallium
MF	magnetic field
MOI	multiplicity of infection (plaque-forming units/cell number)
MR	migratory restlessness behaviour
MV	ventral mesopallium
N	nidopallium
nBOR	nucleus of the basal optic root
ONL	outer nuclear layer
OPL	outer plexiform layer
OT	optic tectum
P	pallidum
P (bla)	promoter for *bla* gene
P (LAC)	promoter (*lac*-operon)
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque-forming unit(s)
PHR	photolyase homology region
pUC ori	pUC-derived DNA replication origin
PVDF	polyvinylidenfluorid
(R)	resistance
R. o. medialis	ramus ophthalmicus medialis
rpm	rounds per minute
RT	reverse transcriptase
SCRP	spin-correlated radical pair
SDS	sodium dodecyl sulfate
Sf	*Spodoptera frugiperda*
SPM	superparamagnetic
St	striatum
ST mixing	Singlet ↔ Triplet radical pair interconversion
T	Tesla (SI unit for magnetic flux density) [1 Gauss (G) = 10^{-4} T]
TA	transient absorption
Trp	tryptophane
U	Unit
UV	ultraviolet
v	ventricle
W	visual Wulst
ZENK	bird acronym; gene is known in other species as *zif-268, egr-1, NGF-IA* and *krox-2*

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Education

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Oct 2001 – Sep 2002	MSc in Integrative Biosciences, Department of Zoology, University of Oxford. Two independent theses: "The nuclear localisation of *aly*" and "Survival rate estimation of the Manx Shearwater, *Puffinus puffinus*". (Graduation: Oct 2002).
Oct 2000 – Sep 2001	Major studies in Biology at the Humboldt University, Berlin. (Theoretical biology, Animal behaviour and physiology, Molecular biology).
Oct 1998 – Sep 2000	Matriculation and Minor studies at the Ruprecht-Karls-University, Heidelberg. (Pre-Diploma, July 2000)
1983 – 1997	Primary and secondary schools. Graduation with Abitur, June 1997, Goethe-Gymnasium Emmendingen.

Additional Experience

Apr 2001	Practical course in behavioural biology at the *Max-Planck Reasearch Institute for Ornithology*, Seewiesen (research group of PD Dr. Biebach) ⇒ Optimised energy and water budget in Barn Swallows, *Hirundo rustica*.
Dec 2000	Excursion with the *Bundesforschungsanstalt für Fischerei*, Cuxhaven ⇒ Fish diseases and parasitism in the North- and Baltic Sea.
Apr 2000	Taxonomically and morphological orientated practical at the research institution *Senckenberg-Museum*, Frankfurt. Department *Crustacea* (research group of PD Dr. Turkay) ⇒ geographical distribution patterns of the genus *Macrobrachia*.
Aug 1999	Ornithological orientated practical at the *Mellum-Rat e.V.* on the island of Wangerooge, Germany. Field assistant, bird monitoring especially during migratory season.
Aug 1998	Participant at an international Work-Camp at the *Peak National Park*, Great Britain ⇒ *"Nature Conservation and Sustainable Development"*.
1998 – 2001	Participant of the *Voluntary Activity Group* of the Visitor Center at the *National Park Schiermonnikoog*, The Netherlands. Conception and guidance of excursions in wadden ecology, saltwater marshes and ornithology.
1997 – 1998	Participant of a FÖJ (voluntary ecological year) at the *Nationalpark-Haus Dornumersiel*, Lower Saxonia. Conservation and environmental education work, bird monitoring (German National Waterbird monitoring programme, International Waterfowl Census).

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Honours

2001 – 2002	DAAD scholarship (German Academic Exchange Service)
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2003 – 2006	*e-fellows.net* online scholarship

Membership

2003 – Present	*Deutsche Ornithologen Gesellschaft* (DOG)
2005 – Present	*Royal Institute of Navigation* (RIN)

Conferences

Sept 2005	Poster presentation at the *Spin Chemistry Meeting 2005*: "Magnetoperception in night-migratory songbirds - a spin chemical mechanism?"
August 2005	Poster presentation at the *350. Heraeus-Seminar - Biophysics of magnetic orientation in animals*: "Cryptochrome, a potential primary magnetoreceptor in night-migratory songbirds".
April 2005	Talk at the *RIN05 - Orientation and Navigation - Birds, Humans and other animals*: "Magnetoreception in night-migratory songbirds a search for a primary receptor".
Februar 2005	Poster presentation at the 6^{th} *German Neuroscience Conference*: "The mystery of magneto-reception in migratory songbirds".

Journal Publications

H. Mouritsen, U. Janssen-Bienhold, **M. Liedvogel**, G. Feenders, J. Stalleicken, P. Dirks & R. Weiler (2004): Cryptochromes and activity markers co-localize in bird retina during magnetic orientation. *PNAS* **101**, 14294-14299.

H. Mouritsen, G. Feenders, **M. Liedvogel** & W. Kropp (2004): Migratory birds use head scans to detect the direction of the Earth's magnetic field. *Current Biology* **14**, 1946-1949.

H. Mouritsen, G. Feenders, **M. Liedvogel**, K. Wada & E.D. Jarvis (2005): A night vision brain area in migratory songbirds. *PNAS* **102**, 8339-8344.

Paper in preparation

M. Liedvogel, G. Feenders, K. Wada, E.D. Jarvis, & H. Mouritsen: Lateralised activation of Cluster N in the brains of migratory songbirds. *submitted*

M. Liedvogel, K. Maeda, K. Henbest, E. Schleicher, T. Simon, P.T. Hore, C.R. Timmel H. Mouritsen: Chemical magnetoreception: bird cryptochromes are exited by light, absorb in the blue spectral range, and form long-lived radicals. *in preparation*

G. Feenders, **M. Liedvogel**, K. Wada, H. Mouritsen & E.D. Jarvis: Movement-driven gene expression patterns implicate origin of brain areas for vocal learning. *under review*

R.H. McCleery, **M. Liedvogel**, J. Stewart & C.M. Perrins: Survival rates of Manx shearwaters *Puffinus puffinus*: comparisons between two populations.*under review*

R. Begum, J.Q. Jiang, **M. Liedvogel** & H. White-Cooper: The transcriptional regulator Mip130 is required for normal function of the cortical actin cytoskeleton. *in preparation*

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Paper I

Mouritsen, H., Feenders, G., Liedvogel, M. & Kropp, W.:

Migratory birds use head scans to detect the direction of the Earth's magnetic field.

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Migratory Birds Use Head Scans to Detect the Direction of the Earth's Magnetic Field

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Summary

Night-migratory songbirds are known to use a magnetic compass [1–3], but how do they detect the reference direction provided by the geomagnetic field, and where is the sensory organ located? The most prominent characteristic of geomagnetic sensory input, whether based on visual patterns [4–7] or magnetite-mediated forces [8, 9], is the predicted symmetry around the north-south or east-west magnetic axis. Here, we show that caged migratory garden warblers perform head-scanning behavior well suited to detect this magnetic symmetry plane. In the natural geomagnetic field, birds move toward their migratory direction after head scanning. In a zero-magnetic field [10], where no symmetry plane exists, the birds almost triple their head-scanning frequency, and the movement direction after a head scan becomes random. Thus, the magnetic sensory organ is located in the bird's head, and head scans are used to locate the reference direction provided by the geomagnetic field.

Results and Discussion

We observed and recorded the behavior of 35 night-migratory garden warblers, *Sylvia borin*, placed individually in a cylindrical orientation cage (Figure 1A), directly to hard disk and/or to video tape by two infrared (840 nm) video cameras (top and side view, inbuilt IR light sources) at night (indoors, light level 0.04 lux from four diffused white light bulbs not directly visible to the birds) or during the day (indoors, room light level 275 lux). Each bird had a thin line of infrared-reflective tape glued to its head, and they were tested inside a windowless wooden house. Seven birds were tested during the day in the natural geomagnetic field. The other birds were tested while showing migratory restlessness at night in the natural geomagnetic field ($n = 11$), a zero magnetic field [10] ($n = 11$) or a changing magnetic field switching 120° every 5 min ($n = 6$). The birds tested at night in the natural magnetic field (NMF) showed magnetic orientation directed in the normal migratory direction (Figure 1B), whereas the birds tested in the zero-magnetic field (ZMF) oriented randomly (Figure 1C). How did the NMF birds detect the compass direction of the geomagnetic field?

The video recordings suggested that birds, in addition to their migratory restlessness behavior, perform repeated head-scanning behavior (see movies in the Supplemental Data available with this article online). A naïve observer (W.K.) counted the number of head scans performed by each individual bird during a 1 hr period. We defined a head scan as the turn of the bird's head from the body axis position to an angle turned clearly more than 60° to the left or right, followed by the subsequent return of its head to the straight-ahead position while the bird remained at the same spot (Figure 1D). By requiring that the bird must return its head to the body axis position before moving in the cage, we avoided counting head turns, which always precede movement in a new direction. The side-view camera showed that the head-scanning behavior was performed in the horizontal plane. Sometimes a bird makes a head scan to one side only; other times, one head scan is immediately followed by another head scan in the opposite direction. Four pieces of evidence strongly suggest that head-scanning behavior is directly involved in the process of sensing the geomagnetic reference direction needed for magnetic compass orientation.

First, garden warblers exposed to a zero-magnetic field (ZMF) made 141 ± 33 (SD) head scans in 60 min, whereas birds tested under any other magnetic condition only made 52 ± 35 (SD) head scans in 60 min (see Figure 1E). The increased head-scanning frequency observed in the ZMF birds is highly significant (one-way ANOVA followed by Tukey all pair-wise comparison method: the ZMF group differs significantly [$p < 0.001$] from all other groups, whereas all other differences between groups are non-significant [$0.22 < p < 0.99$]). On average, the birds, irrespective of magnetic condition, performed an equal number of head scans to the left and to the right (mean = 50% ± 18% [SD]). We also quantified the number of flights and wing beats performed by each bird during the same 60 min period, but we found no significant differences depending on the magnetic field condition (flights: one-way ANOVA on ranks, $p = 0.99$, wing beats: one-way ANOVA, $p = 0.77$). Thus, differences in head scan frequency are not due to differences in activity level between NMF and ZMF birds.

Second, garden warblers strongly increased their head-scanning frequency before initiating their first migratory restlessness behavior (repeated jumping and wing flapping on the perch). Birds observed in the orientation cages typically sat still for 10–60 min after the lights went off. During the last 10 min before initiating their first migratory restlessness behavior that night, all birds made many repeated head scans. Within this period, the average head scan frequency increased gradually from ~2/min to about ~6/min (Figure 2A). This strong increase in head-scanning frequency suggests that the birds carefully determined the reference direction of the geomagnetic field before starting their orientation behavior. Once migratory restlessness behavior was initiated, birds experiencing the NMF made less than one head scan per minute on average, whereas birds experiencing the ZMF performed two to three head scans

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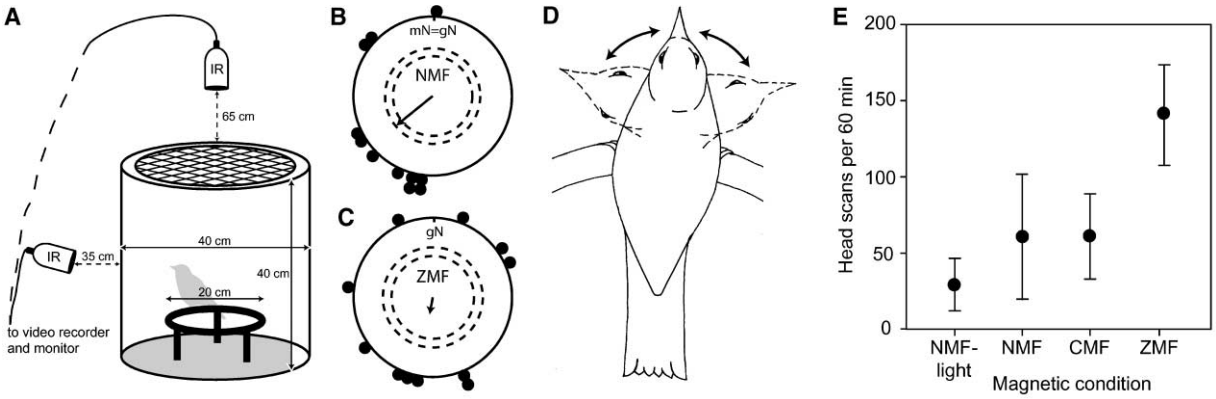

Figure 1. Caged Garden Warblers Perform Head Scan Behavior during Magnetic Orientation

(A) Design of our orientation cage.

(B) Garden warblers tested in the natural magnetic field oriented in their southwesterly migratory direction ($\alpha = 231°$, $r = 0.60$, $p < 0.02$). Each dot indicates the mean orientation of one individual garden warbler (measured as its head's location relative to the center of the cage determined 5 times per s during 45–60 min of constant migratory restlessness behavior). The arrow indicates the group's mean vector length (r). The inner- and outer-dashed circles indicate the length of the group's mean vector needed for significance ($p < 0.05$ and $p < 0.01$, respectively), according to the Rayleigh test. mN = magnetic North; gN = geographic North.

(C) Garden warblers tested in a zero-magnetic field oriented randomly ($\alpha = 187°$, $r = 0.22$, $p = 0.60$).

(D) Schematic drawing of head-scanning behavior.

(E) Number of head scans performed by garden warblers within 60 min under four different conditions: during the day in the natural magnetic field (NMF-light) and while showing migratory restlessness at night in the natural magnetic field (NMF), a changing magnetic field (CMF), or a zero magnetic field (ZMF).

per minute (Figures 1E and 2B). The fact that the birds continued to perform regular head scans throughout the night, even in the natural magnetic field, suggests that they have not transferred magnetic information to other cues in their cage or surroundings.

Third, during migratory restlessness behavior, most garden warblers made occasional flights to the top of the cage followed by fluttering around and landing on the bottom of the cage. After sitting at the bottom of the cage for a few seconds, most birds returned to the perch, where they usually sat still for 10–60 s before reinitiating migratory restlessness behavior. During this period of sitting still after a flight, the birds seem to reorient themselves before continuing their migratory restlessness behavior. This is evidenced by a highly significant 2-fold increase in head-scanning frequency during the first minute following a flight off the perch compared with any other 1 min period (176 head scans observed in 93 1 min periods immediately after a flight compared to 225 head scans observed in 238 other 1 min periods; chi-square test: $df = 1$, $\chi^2 = 49.5$, $p < 0.001$).

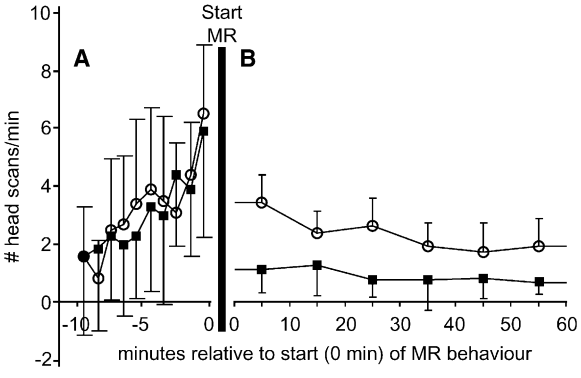

Figure 2. Number of Head Scans Performed per Minute Just before and during Migratory Restlessness Behavior

(A) Just before initiation of their first migratory restlessness behavior at night, garden warblers strongly increase their head-scanning frequency. On the x axis, "0" indicates the time when a bird performed its first migratory restlessness behavior. The symbols are as follows: ■: NMF birds; ○: ZMF birds.

(B) During migratory restlessness behavior, birds experiencing a ZMF continue to show a relatively high head-scanning frequency (two to three head scans per minute), whereas birds experiencing NMF conditions only make about one head scan per minute on average. Error bars indicate (symmetrical) standard deviations.

Fourth, if head scans indeed help garden warblers detect the reference compass direction provided by the geomagnetic field, one should expect that the birds in the natural magnetic field move more toward than away from their mean migratory direction after performing a head scan, whereas the direction of movement after a head scan in a zero-magnetic field should be close to random. We tested this by observing the garden warblers' very first move immediately after they performed a head scan. This was done by placing an arrow on the TV monitor pointing in the overall mean direction of the individual bird. Then, if a bird with a mean orientation of 205°, for example, performs a head scan while sitting on the circular perch at 140°, a clockwise move along the perch would be toward the "correct" direction, whereas a counter-clockwise move would be counted as a move in the "wrong" direction. If it performs a head scan at 265°, a counter-clockwise move would be correct and a clockwise move would be wrong. For both the NMF and ZMF condition, we only analyzed birds that showed an overall mean direction oriented in the appropriate, southwesterly mean migratory direction (215° ± 60°) characteristic for garden warblers during autumn migration. Otherwise, no clear correct migratory direction

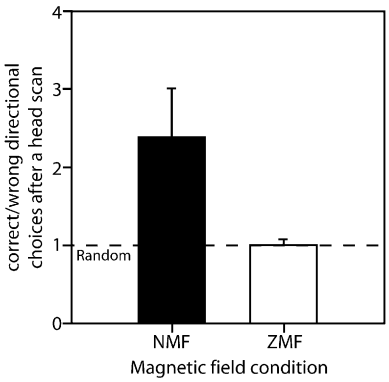

Figure 3. Orientation of NMF and ZMF Birds Immediately Following a Head Scan

Birds experiencing a natural magnetic field move 2.39 ± 0.63 times as often toward their correct migratory direction than away from it after performing a head scan. In contrast, birds experiencing a zero-magnetic field move equally often toward and away from their mean migratory direction following a head scan. Error bars indicate standard deviations.

could be defined. Furthermore, to be reliably analyzed, a bird must have performed most of its migratory restlessness behavior on the perch, where one can clearly determine if the next movement following a head scan is in the correct or wrong direction. All the garden warblers performing migratory restlessness behavior on the perch in the natural magnetic field moved significantly more toward their mean migratory direction after performing a head scan than away from it (number of correct moves/number of wrong moves = 2.39 ± 0.63, Figure 3), whereas the direction of movement following a head scan in a zero-magnetic field was random (number of correct moves/number of wrong moves = 1.01 ± 0.06; difference between NMF and ZMF birds: Mann-Whitney U-test, $p < 0.01$; Figure 3). This difference cannot be explained by a difference in individual directedness (r values) between the ZMF (0.19 ± 0.12) and NMF (0.24 ± 0.18) birds because this difference was nonsignificant (t test, $p = 0.67$).

Based on these converging pieces of evidence, we suggest that caged garden warblers, and possibly night-migratory birds in general, use head movements to detect the reference compass direction of the earth's magnetic field. If magnetoreception in birds is magnetite mediated [8, 9], we suggest that the head movements are designed to scan for the maximum or minimum magnetic field strength direction. If magnetoreception is vision dependent [4–7], we suggest that the purpose of the head scans is to detect the symmetry axis of the magnetically modulated visual patterns that characterize the magnetic-field axis [5] and/or to improve detection of these gradually changing patterns by the visual system, which is more sensitive to them when they move. In fact, due to the predicted graded nature of the magnetically modulated, virtual visual patterns, they may very well be undetectable unless the bird moves its eye relative to the pattern [11]. We also suggest that the strongly increased head-scanning frequency observed in ZMF-birds during migratory restlessness behavior is a result of their repeated, unsuccessful attempts to find a symmetry plane or pattern that does not exist.

The fact that head scanning seems to be used by garden warblers to detect the compass direction of the geomagnetic field confirms that their magnetic sensor must be located in the head. If head scanning is performed by all birds, this will remove an important uncertainty in our search for the avian magnetic sensor and would provide crucial knowledge when designing magnetic-manipulation devices for free-flying birds [12]. Furthermore, virtually all psychophysical experiments designed to elucidate the functional characteristics of the avian magnetic compass have been unsuccessful [13]. We suggest that head scan counts can be used as a much-needed psychophysical measure to determine many unknown functional characteristics of the avian magnetic compass.

Supplemental Data

Supplemental Data including two movies are available at http://www.current-biology.com/cgi/content/full/14/21/1946/DC1/.

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Paper II

Mouritsen, H., Janssen-Bienhold, U., Liedvogel, M., Feenders, G., Stalleicken, J., Dirks, P. & Weiler, R.:

Cryptochrome and neuronal-activity markers colocalize in the retina of migratory birds during magnetic orientation.

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Cryptochromes and neuronal-activity markers colocalize in the retina of migratory birds during magnetic orientation

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Migratory birds can use a magnetic compass for orientation during their migratory journeys covering thousands of kilometers. But how do they sense the reference direction provided by the Earth's magnetic field? Behavioral evidence and theoretical considerations have suggested that radical-pair processes in differently oriented, light-sensitive molecules of the retina could enable migratory birds to perceive the magnetic field as visual patterns. The cryptochromes (CRYs) have been suggested as the most likely candidate class of molecules, but do CRYs exist in the retina of migratory birds? Here, we show that at least one CRY1 and one CRY2 exist in the retina of migratory garden warblers and that garden-warbler CRY1 (gwCRY1) is cytosolic. We also show that gwCRY1 is concentrated in specific cells, particularly in ganglion cells and in large displaced ganglion cells, which also showed high levels of neuronal activity at night, when our garden warblers performed magnetic orientation. In addition, there seem to be striking differences in CRY1 expression between migratory and nonmigratory songbirds at night. The difference in CRY1 expression between migrants and nonmigrants is particularly pronounced in the large displaced ganglion cells known to project exclusively to a brain area where magnetically sensitive neurons have been reported. Consequently, cytosolic gwCRY1 is well placed to possibly be the primary magnetic-sensory molecule required for light-mediated magnetoreception.

Since the description of animal magnetosensory capabilities in the 1960s (1–3), it has been convincingly shown that songbirds can use a magnetic compass for orientation during their migratory journeys (3–6), but the physiological mechanisms enabling migratory birds to sense the reference direction provided by the Earth's magnetic field still remain unknown. Two types of potential magnetoreception mechanisms have been suggested over the past decades: one mechanism that is based on magnetite particles and one mechanism that is based on photoreceptors forming radical-pair intermediates (for summary, see ref. 7). Although no direct physiological or molecular evidence has been reported, numerous orientation cage experiments with captive migratory songbirds have revealed several important characteristics of their magnetic compass.

The magnetic compass of migratory songbirds is an inclination compass; that is, it detects the axis but not the polarity of the magnetic field lines (4–5). Furthermore, magnetic orientation in migratory songbirds depends on the wavelength of the ambient light (8–11). Migratory songbirds are active and orient magnetically under dim blue and green light, whereas they are active but disoriented under dim red light (8–10). These findings strongly suggest that photoreceptor molecules in the eye are involved in magnetoreception and that these photoreceptor molecules should absorb in the blue and green range of the spectrum. The involvement of photoreceptors in the eye is further supported by the finding that birds with their right eye covered seem unable to perform magnetic orientation (12). A recent behavioral experiment (13) testing the magnetic orientation responses of European robins, *Erithacus rubecula* (a night-migrating songbird), exposed to oscillating magnetic fields provided strong indirect evidence that the magnetic-inclination compass of night-migrating songbirds is based on a radical-pair mechanism (7, 13).

Photoreceptor-based radical-pair mechanisms were suggested by Schulten *et al.* (14) and strongly elaborated on by Ritz *et al.* (7). They are based on the fact that radical-pair reactions will be modulated differently depending on the direction of the Earth's magnetic field relative to the orientation of the radical-pair-forming molecule (7, 15, 16). In short, the current hypothesis (7) further suggests that light in the blue-green range will excite photoreceptors forming radical pairs upon photoexcitation in the retina of the migratory bird. Because of the shape of the retina (half ball) and the presumed fixed orientation of the radical-pair-forming photoreceptors inside the cells, the magnetic field would modulate the radical-pair reaction and, thereby, the light sensitivity differently in different parts of the retinas, leading to perception of the magnetic field as visual patterns (7). Radical-pair-mediated magnetoreception would not be able to detect the polarity of the field lines, but only their axis, which is in line with the inclination-based nature of the songbird magnetic compass (4, 10).

Based on these theoretical considerations and behavioral evidence, the primary magnetic-sensory molecule in the retina of migratory songbirds should be a photopigment that is excited by light in the blue-green range and forms radical pairs upon photoexcitation. The cryptochromes (CRYs) (17–22) have been suggested as the most likely candidate class of molecules (7) because they are blue-green photoreceptors in plants (17, 19, 22) and because closely related 6,4-photolyases have been shown to form radical pairs upon photoexcitation (23). Other classes of photoreceptors, such as phototropsins (24) and chlorophylls (25), found in plants can also undergo radical-pair reactions. Rhodopsins should not be able to form radical pairs because photoexcitation leads to cis–trans isomerization of retinal rather than an electron transfer (e.g., ref. 26). Thus, CRYs are the only currently known class of molecules found in the retina of vertebrates that are likely to fulfill the physical and chemical characteristics that are required for function as the primary magnetic sensor (7).

Therefore, the aims of the present article are to (*i*) test whether CRYs exist in the retina of migratory birds performing a magnetic orientation task at night, (*ii*) elucidate their cellular location within the retina, (*iii*) test whether the CRY-containing cells show neuronal activity when migratory birds perform night-time magnetic orientation behavior, and (*iv*) compare the

Abbreviations: CRY, cryptochrome; gwCRY, garden-warbler CRY; INL, inner-nuclear layer.

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results of the first three aims in migratory songbirds with a nonmigratory songbird.

Materials and Methods

The migratory behavior of night-migratory songbirds can be studied in caged birds because they are so eager to migrate that they will show migratory-restlessness behavior (wing-flapping and jumping on the perch) that is oriented in the direction in which they want to fly (27–29). In the present study, we tested 30 migratory garden warblers, *Sylvia borin*, and 10 nonmigratory zebra finches, *Taeniopygia guttata*, in orientation cages. The birds were kept indoors in a wooden laboratory under the natural light–dark cycle. At approximately noon, birds that were to be tested were marked with a small stripe of reflective tape on the head and transferred to a circular orientation cage. The timed lights went off at local sunset, reducing the light level in the room to 0.04 lux, originating from four diffused white light bulbs that were not directly visible to the birds. The behavior of the birds inside the orientation cage was monitored by an infrared (840 nm) video camera. The orientation of the birds was tested in the undisturbed geomagnetic field (field strength, 48,300 nT; inclination, 67°) or in an equivalent magnetic field (field strength, 48,300 ± 200 nT; inclination, 67° ± 0.2°) turned 120° ± 0.2° in the horizontal plane by 3D (2 × 2 × 2 m) Helmholtz coils (28).

During these orientation experiments, we carefully observed the birds on a video screen in real time and collected the retinas of 11 garden warblers immediately after they had shown at least 1 h of consistent migratory-restlessness behavior. All birds spent ≥100 min in the orientation cage after the lights went off before being killed. We also collected retinas from four garden warblers in room-light conditions in which they do not show migratory behavior. As nonmigratory controls, we collected the retinas of 10 zebra finches (five during the day and five at night).

When a given bird had shown ≥1 h of migratory-restlessness behavior (garden warblers at night; garden warblers during the day and all zebra finches just had to stay awake), it was killed according to the German legislation regulating the use of animals in research. Actual collection times varied between 22:23 and 00:40, equivalent to between 1 h and 40 min and 4 h after the lights went off. The day birds were collected between 19:48 and 20:30 having spent the preceding 12–14 h under constant room-light (275 lux) conditions. In all cases, eyecups were prepared within 6–13 min of the death of the bird under dim red light and were fixed in 2% paraformaldehyde, embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), and frozen to −80°C.

For the detection of CRY1 transcripts, total RNA was extracted from the retinas by means of a NucleoSpin RNA II Kit according to the manufacturers protocol (Macherey & Nagel), which included treatment with DNase I to exclude contamination with genomic DNA. cDNA synthesis was performed from either 2 μg of RNA with SuperScript II Rnase H^- reverse transcriptase (Invitrogen) or from ≈700 ng of RNA with Revert AID H^- First-Strand cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturer's protocols. First-round hot-start PCRs (total volume, 25 μl) included 2 μl of cDNA/1× reaction buffer (Promega)/1,25 mM $MgCl_2$/0.2 mM each dNTP (Roche Diagnostics, Mannheim, Germany)/0.8 μM each primer (MWG Biotech, Ebersberg, Germany)/1 unit of *Taq* polymerase (Promega). cDNA synthesis and contamination with genomic DNA was controlled by using intron-spanning β-actin-specific primers [USP, 5′-GGCATGTGCAAGGCCGGCTTC-3′ (exon 2); and DSP, 5′-GGATGGCATGAGGGAGCGCGT-3′ (exon 4)] with the following conditions: 2 min at 95°C; 35 cycles of 30 s at 95°C, 1 min at 64°C, and 1.5 min at 72°C; and 15 min at 72°C). For the detection of the 642-bp CRY1 transcripts in the left and right retina, degenerate primers [USP, 5′-TTGTCGACAATGCTGGAAG(CT)TGGA-3′; and DSP, 5′-TTGAATTCTTCTTC(CT)(GT)GA(CT)T(AT)G-G(AG)CG-3′] were used with the following conditions: 3 min at 94°C; 40 cycles of 30 s at 94°C, 30 s at 59°C, and 1 min at 72°C; and 2 min at 72°C). The entire coding region of garden-warbler CRY1 (gwCRY1) cDNA was obtained by means of long PCR (3 min at 94°C; and 40 cycles of 45 s at 94°C, 45 s at 54°C, and 2 min at 72°C) with 2 units of Taq plus 0.04 plaque-forming units of polymerase (Stratagene) and the two following additional sets of specific primers: USP^N, 5′-AGCAAGGTCTCCTTTCATCCTCTCAATATTCAGA-3′; DSP^N, 5′-AGCACGGTCTCCCATGGGGGTGAACGCCGTGCACTGGTT-3′ (amplification of the 5′ end); USP^C, 5′-AGCAAGCGGCCGCTTATCAATTTGTGCTCTGCCGCTGGACTTT-3′; and DSP^C, 5′-AGCACGGTCTCCGAAACAGATCTACCAGCAGCTT-3′ (amplification of the 3′ end). The latter primers were designed to get overlapping fragments encoding the N- and C-terminal regions of CRY1, which were then used to reconstruct the full-length gwCRY1 coding sequence. The same procedures were applied to detect a 237-bp fragment of CRY2 by using the following primer pair: USP, 5′-ACGAAGAATTCGACGAAAGCCACATCCAG-3′; and DSP, 5′-AGCACGTCGACGAACCCCATCTGCATCCA-3′. cDNA encoding the C-terminal part of CRY2 (777 bp) was obtained by means of nested PCR using the following primer pairs: USP, 5′-TTATGAGGCCCCCGATTTTCTGTTG-3′; DSP, 5′-AATCCCCTGAGACAGGAACCCTGAAGCC-3′; USP^{nest}, 5′-GTTCCTGCTCCTCTGGTCACCTCTT-3′; and DSP^{nest}, 5′-AGGAACCCTGAAGCCTTGGCAAAG-3′. All cloned fragments were subjected to nucleotide sequencing, and DNA similarities and identity scores were analyzed by FASTA and WU-BLAST2 algorithms.

Frozen cryostat sections (12–15 μm) of the retinas, which were mounted on gelatin-coated slides, were used for immunohistochemical labeling with CRY1 antibodies directed against the near-C-terminal part of the CRY1-protein by following standard protocols (30). Primary-antibody incubation was carried out overnight at 4°C for CRY1/sc-5953 (diluted 1:100), Egr-1/sc-189 (diluted 1:500), and c-Fos/sc-253 (diluted 1:200/1:500). All antibodies were purchased from Santa Cruz Biotechnology and diluted in PBS/0.3% TX-100. Immunoreactivity was visualized by indirect immunofluorescence using FITC-conjugated donkey anti-goat IgG for CRY1 detection and Cy3-conjugated donkey anti-rabbit IgG for Egr-1 and c-Fos detection. In control experiments, primary antibodies were omitted or preabsorbed with the appropriate inhibitory peptide (sc-5953 P, sc-189 P, or sc-253 P), which was diluted 1:50 in the incubation buffer. In double-labeling experiments, incubations with either both primary or both secondary antibodies were carried out at the same time. Labeled sections were analyzed by using a TSC confocal microscope (Leica, Nussloch, Germany), and brightness and contrast of the images were adjusted to the same background level by using PHOTOSHOP software (Adobe Systems, San Jose, CA). The immunosignal intensity was quantified relative to background intensity at the confocal microscope by encircling relevant areas on retina slices from different experimental groups incubated on the same slide, such that the intensity index = {[2× immunostaining intensity (ISI) in ganglion cells/(ISI in inner plexiform + ISI in GCaxon layer)] −1}. Specificity of the antibodies was tested by means of immunoblotting using standard protocols (30). Immunoreactive proteins were detected by using the chemiluminescence method (ECL-system; Amersham Pharmacia Buchler, Braunschweig, Germany). The Western blot analyses were replicated four times with different birds and positive rat-brain controls. The strengths of the bands were in line with the immunosignal quantifications. In control experiments, the primary antibody (diluted 1:500 in PBS) was preadsorbed by its inhibitory peptide (diluted 1:50).

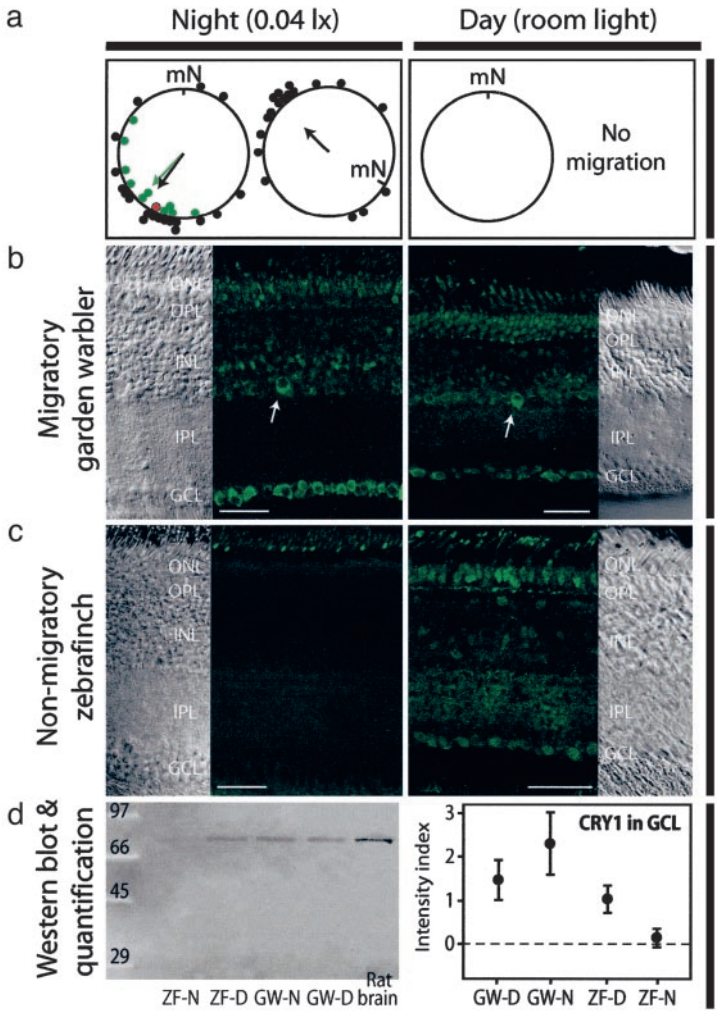

Fig. 1. CRY protein expression in the retina of migratory garden warblers and nonmigratory zebra finches. (*a*) Magnetic orientation of garden warblers. Each data point indicates the mean orientation of one individual. The arrows indicate group mean vector lengths (*r*). The orientation of the night-birds (Night) from which retinas were collected (green symbols) were highly oriented in the normal migratory direction ($n = 11$, mean = 223°, $r = 0.77$, and $P < 0.001$). The red dot represents the night bird whose retina is shown in *b*. During the day, the birds do not show orientation behavior (Day). mN, Magnetic North. (*b*) Immunohistochemical staining of CRY1 protein during nocturnal magnetic orientation (*Left*) and during the day (*Right*) in migratory garden warblers (images from the same slide taken with identical settings). Large displaced ganglion cells marked with arrow. (*c*) Immunohistochemical staining of CRY1 protein during the night (*Left*) and day (*Right*) in nonmigratory zebra finches (images from same slide taken with identical settings). Labeling of photoreceptor outer segments and Müller cell end-feet in *b* and *c* is unspecific. (*d*) Example of Western blot analysis confirming specificity of the antibody and quantification of CRY1 expression in the ganglion cell layer. For definition of intensity index, see *Materials and Methods*. Dashed line indicates unspecific background level of expression. GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer. (Scale bar, 40 μm.)

Results

The magnetic-orientation responses of 30 migratory garden warblers were tested at night in circular orientation cages inside a wooden laboratory. The birds showed clear magnetic orientation (Fig. 1*a*) in their species-specific migratory direction (normal field: mean = 215°, $n = 30$, $r = 0.62$, and $P < 0.001$), and they significantly (Mardia–Watson–Wheeler test; $n = 48$, $\chi^2 = 19.6$, and $P < 0.001$) changed their orientation as predicted (mean = 316°, $n = 18$, $r = 0.51$, and $P < 0.01$) in a turned magnetic field (magnetic North = 120°).

CRY expression at the mRNA level was first analyzed by RT-PCR. First, a gwCRY1 fragment of ≈642 bp (Fig. 2), including the region encoding the FAD-binding region thought to be involved in the radical-pair reaction (18, 23), was amplified with degenerated CRY1 primers. Subsequently, the complete coding sequence was reconstructed (GenBank accession no. AJ632120), and FASTA and WU-Blast2 analysis of the gwCRY1 nucleotide sequence revealed the highest homology (93%) with chicken CRY1 (GenBank accession no. AY034432). We also amplified a 777-bp fragment encoding the C terminus of gwCRY2 (GenBank accession no. AY739908), which showed highest homology (89%) with chicken CRY2 (GenBank accession no. AY034433). Both gwCRYs belong to the animal CRY family (31). Subsequent immunohistochemistry revealed that gwCRY1 was located in the cytosol, whereas gwCRY2 was located in the nucleus. Because a CRY working as the primary magnetoreceptor must be oriented in the cells and, therefore, is most likely linked to cytosolic skeleton proteins, a nucleic location is very unlikely. For these reasons, we focused subsequent experiments on gwCRY1 only.

The specificity of the CRY1 antibodies was confirmed by four independent sets of immunoblots [all showing only one immunoreactive protein at the appropriate molecular mass of ≈70 kDa (Fig. 1*d*)], which disappeared when the antibody was preabsorbed with the corresponding peptide. In migratory garden warblers, immunolabeling for CRY1 was found in specific cell populations across the retina. Invariably, 95–100% of garden-warbler ganglion cells showed high levels of cytosolic CRY1 with a tendency toward higher expression in night birds than in day birds (t test, df = 8, $t = 2.06$, $P = 0.07$; Fig. 1 *b* and *d*). All large, displaced ganglion cells were also strongly labeled for CRY1 in garden warblers in both day and night. The photoreceptor layer of garden warblers showed high expression of CRY1 particularly in day birds. In the inner-nuclear layer (INL) of garden warblers, 10–15% of the cells were CRY1-positive, and night garden warblers tended to show higher levels of CRY1 in a cell population with elongated somata. In nonmigratory zebra finches, the expression in day birds closely resembles that found in garden warblers during the day, except that the large displaced ganglion cells were always CRY1-negative in zebra finches (Fig. 1*c*). However, during the night, CRY1 expression in nonmigratory zebra finches dropped dramatically to nonspecific background level (night mean of intensity index [(signal/background) − 1] = 0.14 ± 0.21 (SD); day mean of intensity index = 1.04 ± 0.31;

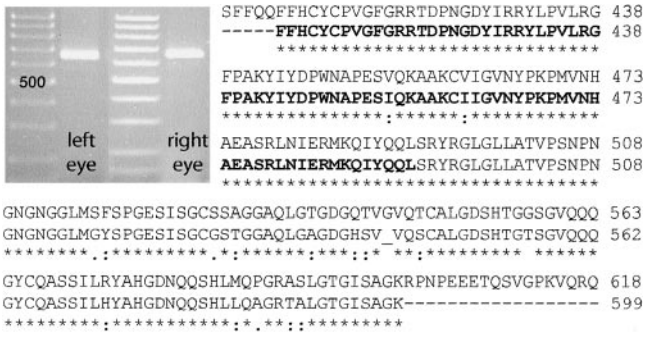

Fig. 2. Detection of a CRY1 transcript with the expected size of ≈642 bp in the left and right retinas of garden warbler (lower sequence). The deduced amino acid sequence for this fragment reveals 91% identity with the specific C-terminal region of chicken CRY1 (upper sequence). The FAD-binding domain thought to be involved in radical-pair reactions is shown in bold. Upper left, 5′ end; lower right, 3′ end.

t test, df = 8, $t = 5{,}35$, and $P < 0.001$; Fig. 1 *c* and *d*). This decrease in CRY1 expression is in line with the normal circadian oscillation found previously in CRY1 mRNA in nonmigratory domestic chicken and quail (20, 21) but strikingly different from the unusually high expression levels [mean intensity index = 2.31 ± 0.72 (SD)] found in migratory garden warblers at night (Fig. 1*d*). There were no obvious differences in CRY1 patterns between the right and left eye (12).

To be potentially relevant for magnetoperception, the CRY-containing retinal cells of garden warblers must be active at night when the birds perform magnetic orientation. We tested the neuronal-activity pattern of retinal cells by using the markers c-Fos and ZENK (*zif*268, *E*gr-1, *N*GF-1A, *K*rox-24). The transcription factors (immediate early genes) c-Fos and ZENK are known to show vision-dependent expression in the retina of many animals (32–35), including chicken (32), and their expression requires neuronal activity (36–37). The c-Fos and ZENK proteins were detectable in a cell 15–20 min after neuronal activity occurred, and peak protein expression was detected after 45–60 min (38). Thus, c-Fos and/or ZENK protein detected immunohistochemically in a cell was produced by neuronal activity 15–90 min before the tissue was fixed, which matches the time frame in which our birds performed magnetic orientation. Consequently, colocalization of c-Fos and ZENK protein in garden-warbler retinal cells showing high CRY levels would show that these cells were sending neuronal information to the brain while our birds oriented to the geomagnetic field at low night light levels (0.04 lux).

As expected, immunostaining and Western blot analysis revealed higher c-Fos expression in both zebra finches (*t* test, df = 8, $t = 7.18$, and $P < 0.001$) and garden warblers (*t* test, df = 8, $t = 3.25$, and $P < 0.02$) during the day, when vision is more active, than during the night (Fig. 3*a*). But whereas c-Fos expression during the day was similar in garden warblers and zebra finches, at night it was significantly higher (*t* test, df = 8, $t = 4.474$, and $P < 0.01$) in the migratory garden warblers. In these night garden warblers, c-Fos is strongly expressed in ganglion cells and in ≈5% of the cells within the INL (Fig. 3*b*). ZENK was strongly expressed in the nuclei of garden-warbler ganglion cells (Fig. 3*c*). The c-Fos and ZENK signals colocalized with CRY1 in all garden-warbler ganglion cells and large displaced ganglion cells but only sporadically in other cell types at night during magnetic orientation (Fig. 3 *b* and *c*).

In their theoretical article, Ritz *et al.* (7) specifically suggested that the large, morphologically distinct (39, 40), and displaced ganglion cells are likely to be a location for magnetoreception because they project exclusively to the nucleus of the basal optic root (39, 40), where magnetically sensitive neurons have been reported (41) and visual flow-fields arising from self-motion are processed (42). The scarce, large CRY1-positive cells found in the INL of the garden warbler (Figs. 3 and 4) share the features reported for displaced ganglion cells in birds (39, 40) (in particular, their large size, disk-shaped nuclei, and large cytosolic space) and, thus, are easy to locate by Nomarski optics. Thy1, a general marker for ganglion cells, also in birds (43), further confirmed their identification as displaced ganglion cells. We analyzed >100 of these large cells in the garden warbler and >30 in the zebra finch, and we found striking differences in CRY1 and c-Fos expression. In the migratory garden warbler (day and night), the large displaced ganglion cells always contained strong CRY1 immunolabeling and a strong label for c-Fos (Fig. 4). In contrast, the large displaced ganglion cells of nonmigratory zebra finches (day and night) were always CRY1-negative (Fig. 4). Furthermore, all of the >20 large displaced ganglion cells that were analyzed in night zebra finches were c-Fos-negative. In zebra finches during the day, as expected because of normal visual processes, both c-Fos-positive (active) and c-Fos-negative (inactive) large displaced ganglion cells were found, but even when active, they never expressed CRY1.

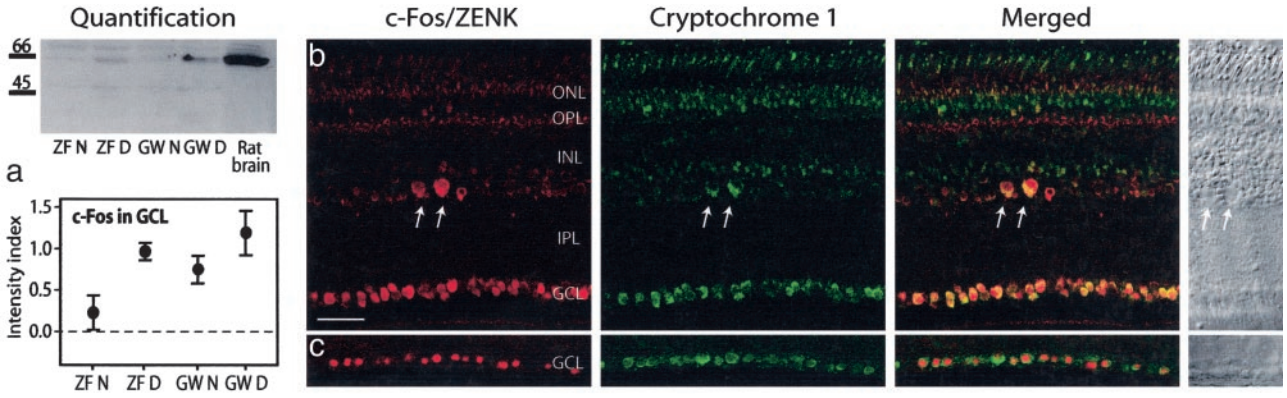

Fig. 3. Colocalization of CRY1 and neuronal-activity markers in the same 300-nm-thick retina slice from a magnetically orienting garden warbler. (*a*) Example of Western blot analysis confirming specificity of the antibody and quantification of c-Fos expression in the ganglion cell layer. For definition of intensity index, see *Materials and Methods*. Dashed line indicates unspecific background level of expression. (*b* and *c*) Immunohistochemical double labeling of CRY1 (cytosolic) with the neuronal-activity markers c-Fos (*b*; cytosolic and nucleic) and ZENK (*c*; exclusively nucleic; only the ganglion cell layer is shown) reveal that CRYs are found in night-active ganglion cells and in large displaced ganglion cells of the INL (arrows). (Scale bar, 40 μm.) GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

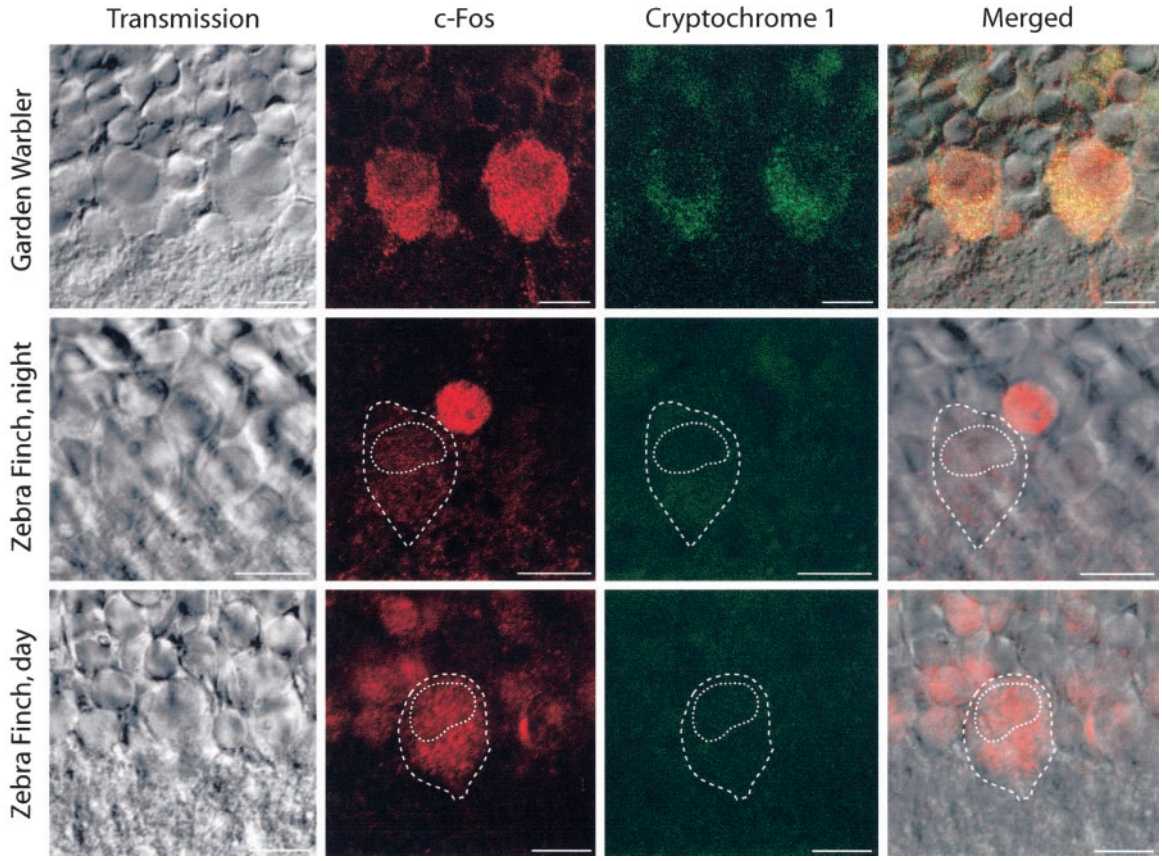

Fig. 4. High magnification confocal images confirm the colocalization of CRY1 with the neuronal-activity marker c-Fos in large displaced ganglion cells in the garden warbler [during both night (shown) and day (data not shown)]. In contrast, the large displaced ganglion cells never express CRY1 in zebra finches, neither during the night nor during the day, when both active (c-Fos-positive, as shown) and inactive (c-Fos-negative) large displaced ganglion cells occur in zebra finches.

Discussion

Although no direct link between CRY-containing cells and magnetoreception is currently known, we present here a number of strong correlations supporting such a link. We have shown that at least one CRY1 exists in the retina of migratory birds and that this gwCRY1 is apparently predominantly or even exclusively located in the cytosol. We have shown that CRY1 is concentrated in specific cells, particularly in ganglion cells and in large displaced ganglion cells, and we have shown that these cells also show high levels of neuronal activity at night during magnetic orientation in migratory garden warblers. That is, the CRY-containing cells in the eye send information to the brain at night when garden warblers orient to the geomagnetic field. Consequently, cytosolic CRY1 is well placed to possibly take over the role of the required substrate for magnetoreception according to the Ritz *et al.* model (7). We also show that there seem to be striking differences in CRY expression between migratory and nonmigratory birds at night. As in domestic chicken, there seems to be virtually no CRY1 in the retina of nonmigratory zebra finches at night, whereas it is strongly expressed in migratory garden warblers at night. The differences in CRY1 expression between migrants and nonmigrants are particularly pronounced in the large displaced ganglion cells that are known to project exclusively to the nucleus of the basal optic root, where magnetically sensitive neurons have been reported (41). Furthermore, the neuronal activity of the ganglion cells is significantly lower at night in nonmigratory zebra finches than in migratory garden warblers, suggesting that the ganglion cells send more information to the brain at night in migratory birds. Because CRYs are found in the retina of both night-migratory and nonmigratory birds during the day, our data do not exclude that a CRY-mediated magnetic compass could be used also during the day, when wavelength-dependent magnetic-compass orientation has also been observed (10).

CRYs in other vertebrates have been shown to be involved in the inhibitory branch of the autoregulatory transcriptional loop controlling the circadian clock (19), whereas the presumed photoreceptor function of vertebrate CRYs is still debated (17, 19). Our data do not exclude a clock function of the gwCRYs, and the high CRY1-expression in night-migratory garden warblers could be a result of their round-the-clock activity during the migratory season. However, garden warblers taken on nights when they do not show migratory restlessness also show high levels of CRY1-expression (H.M., U.J.-B., M.L., G.F., and R.W., unpublished data), and the cytosolic localization of CRY1 that we found in the garden warbler does not seem to favor a role in the clock transcriptional loop because these processes occur in the nucleus (17).

In conclusion, our results support the hypothesis that CRYs, in addition to their circadian functions (17–22), could indeed play a role in magnetic field reception. Having made this statement, we stress that there are still many questions that must be answered before we can state conclusively that the CRYs are the primary magnetic sensor in migratory birds. Although it has recently been shown that the radical pair of 6,4-photolyase, which is highly homologous to the CRYs, live long enough (>5 μs) for geomagnetic field effects to take place (44), it, for instance, still needs to be shown how the orientation of the CRYs is fixed in cells so that light-induced signals will be modulated

differently by the magnetic field depending on location in the retina. However, the location of gwCRY1 in the cytosol means that it could be fixed to the cytoskeleton. In any case, having identified the CRY-containing cells in the retina opens a number of experimental possibilities, which we believe will ultimately lead to an understanding of the molecular and physiological mechanisms underlying magnetic-compass orientation in migratory birds.

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Paper III

Liedvogel, M., Maeda, K., Henbest, K., Schleicher, E., Simon, T., Hore, P.J., Timmel, C. R. & Mouritsen, H.:

Chemical magnetoreception: bird cryptochromes are excited by light, absorb in the blue spectral range, and form long-lived radicals.

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Chemical magnetoreception: bird cryptochromes are excited by light, absorb in the blue spectral range, and form long-lived radicals

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Key words: light-mediated magnetoperception, radical pair process, magnetic compass orientation, garden warbler, *Sylvia borin*

Abstract

Cryptochromes (Cry) have been suggested to form the basis of magnetic compass orientation in birds. However, to function as magnetic compass sensors, cryptochromes of migratory birds must fulfil a number of biophysical characteristics. Most importantly they must absorb light and form long-lived radical pairs. Here we recombinantly expressed and purified two versions of Cryptochrome 1a from the migratory garden warbler (*gw*Cry1a) as flavoproteins from a baculovirus/*Sf9* cell expression system. We used these garden warbler cryptochromes to experimentally verify that they are excited by light in the blue spectral range and that this light-excitation leads to formation of radical pairs that are very long-lived (milliseconds). These biophysical characteristics make *gw*Cry1a ideally suited as primary light-mediated, radical-pair-based magnetic compass receptors.

Introduction

One of the most fascinating unresolved mysteries of sensory biology is the question how migratory birds sense the compass direction provided by the Earth's magnetic field. Migratory birds are known to use the Earth's magnetic field to orient during their long migratory journeys (Wiltschko & Wiltschko 1972, 1995; Cochran *et al.* 2004). Their ability to perform magnetic compass orientation in the lab has been shown to be affected by the wavelengths of the ambient light (Wiltschko *et al.* 1993, 2004) and by covering up one eye (Wiltschko *et al.* 2002), whereas pinealactomised birds (Schneider *et al.* 1994) and birds with their trigeminal nerve (suggested to be involved in magnetite mediated magnetoreception, Fleissner *et al*. 2003; Mora *et al.* 2004) cut are still able to use their magnetic compass to orient in the lab (Beason & Semm 1996). This led to the suggestion that a photolytic process in the eye could produce so-called spin-correlated radical pairs (SCRPs). In favourable circumstances (such as a sufficiently long life time of the radical species) the recombination reactions of SCRPs can be shown to be magnetic field sensitive (Salikhov *et al.* 1984; Steiner & Ulrich, 1998) and hence, it was suggested that a SCRP might function as the primary magnetic compass detector (Schulten *et al.* 1978).

SCRPs are typically the result of a photochemical reaction creating the radicals from a molecular precursor (eg. through homolytic bond-cleavage) in a spin-correlated electronic Singlet (S) or Triplet (T) state. Under the influence of intramolecular nuclear-electron interactions, the radical pair can interconvert between the S and T states, a process known as

Singlet ↔ Triplet interconversion (or ST mixing). Externally applied static and/or oscillating magnetic fields can affect this ST mixing and hence alter the relative ratio of S and T radical pairs. If S and T radical pairs, upon reencounter, react to give different recombination products, applied magnetic fields can affect the yields of S and T products. However, in order for a magnetic field to have a substantial effect on the ST mixing process, the radical pair needs to be sufficiently long-lived (for a field similar to that of the Earth, the radical pair lifetime should typically exceed 1 μs; Cintolesi *et al.* 2003).

Despite encouraging theoretical hypotheses (Ritz *et al.* 2000) and supporting calculations (Timmel *et al.* 2001; Cintolesi *et al.* 2003) there is no experimental proof as yet that the recombination yields of such radical pair reactions are sensitive to the *relative orientation* of the pair within the external magnetic field. This directional information is of course of pivot importance in the discussion of magnetoreception in migratory birds.

However, magnetic fields oscillating with frequencies in the MHz range (Woodward *et al.* 2001, Henbest *et al.* 2004, Rodgers *et al*. 2005) are known to affect the singlet-triplet interconversion processes in SCRPs even in the absence of external magnetic fields. If the magnetic compass of migratory birds were based on a radical pair reaction mechanism, weak magnetic fields oscillating at frequencies in the MHz range might be expected to disrupt the birds' orientation performance (Ritz *et al.* 2000, Henbest *et al.* 2004). This prediction has been experimentally verified by Ritz and colleagues (Ritz *et al.* 2004; Thalau *et al.* 2005); further strengthening the hypothesis that a radical pair reaction is involved in the magnetic compass of migratory birds. Cryptochromes which exhibit a close homology to photolyases (flavoproteins involved in the repair of UV light induced DNA damage) were first proposed by Ritz *et al.* (2000) as the proteins providing the crucial radical pair cofactors to putatively act as primary magnetoreceptors. So far it could be shown that cryptochromes exist in the eyes of migratory birds (Mouritsen *et al.* 2004a; Möller *et al.* 2004) and that at least some cryptochrome-containing cells within the retina are active during night, when the birds performed magnetic orientation in the lab (Mouritsen *et al.* 2004a). It has also been shown that a distinct part of the forebrain is highly active when migratory birds perform magnetic orientation in the lab and that this brain cluster primarily processes input from the eye (Mouritsen *et al.* 2005). All these findings are in line with the hypothesis that magnetic compass detection in migratory birds takes place in the eye and that cryptochromes could be the primary magnetoreceptors. However, several fundamental biophysical characteristics assumed for the primary receptor molecule in the model calculations have not been experimentally validated for any vertebrate

cryptochrome (Mouritsen & Ritz 2005). The aim of this study is to experimentally test the biophysical properties of cryptochromes of migratory garden warbler.

Methods

Cloning. For detection and cloning of garden warbler Cry1 transcripts, total RNA was extracted from the retina of a garden warbler performing orientation behaviour during the migratory season as described in Mouritsen *et al.* 2004. cDNA synthesis was performed from 450 ng of RNA with Revert AID H$^-$First-Strand cNDA synthesis kit (MBI Fermentas) according to the manufacturer's protocol.

Hot start PCR amplification reactions were performed in 25 µl total reaction volume containing 1 µl of the cDNA synthesis reaction as template, 1x reaction buffer (SAWADY/Amersham-Pharmacia, depending on DNA polymerase used), 0.25 mM dNTPs (Pharmacia Biotech), of each primer 1.25 pmol/µl PCR volume, 1 unit of *Pwo* polymerase (SAWADY) or 2 units of *Taq* polymerase (Amersham-Pharmacia). The PCR reaction was carried out in a MWG Primus25 thermocycler. The temperature profile for all hot-start PCR reactions was as follows: first denaturation 3 min 94° C; 45 cycles of 45 s at 94° C, 45 s annealing at 54° C and 40 s extension at 72° C (duration of extension phase depends on the expected size of the amplified fragment and enzyme; extension step comprised 1 min/kb DNA for amplification with *Taq* polymerase and 2 min/kb DNA for *Pwo* amplification); the last cycle was extended to 5 min at 72° C to ensure completion of the final extension. The full length sequence of garden warbler Cry1a (GenBank accession no. AJ632120) was reconstructed as described in Mouritsen *et al.* (2004).

To probe for potential influences of the C-terminal extension of *gw*Cry1a in the reaction process, we additionally expressed the photolyase homology region (510 aa) of garden warbler cryptochrome 1 (Cry1-PHR). The PHR lies within the N-terminal domain, which is identical for both *gw*Cry1a and *gw*Cry1b, and is similar to photolyases and highly conserved in all members of the photolyase/cryptochrome superfamily (Brautigam *et al.* 2004; for a review see Lin & Todo 2005). For expression of a garden warbler Cry1 photolyase-homologous region (Cry1-PHR) the N-terminal 510 amino acids were chosen based on the structural analysis of *A. thaliana* Cry1-PHR (Brautigam *et al.* 2004) and the lack of homology between garden warbler and *A. thaliana* Cry1 beyond amino acid residue 510. A C-terminal fragment for Cry1-PHR

was PCR-amplified from a cloned Cry1a template with the primer pair: DSP^{1PHR} *p741*), 5'-TAGTTCCTTCTTTCAGCAGTTT-3'; USP^{1PHR} (*p742*), 5'-AGCAAGCGGCCGCTTATCAA GTTGCAAGAAGACCCAGTCCT-3'. The amplified fragment (320 bp) was digested with *BstXI/NotI* (resulting in a 107 bp fragment) and cloned with a 1408 bp N-terminal fragment isolated from a full-length Cry1a plasmid by *NcoI/partial BstXI* digest of the full length garden warbler Cry1a plasmid.

Coding regions for *gw*Cry1a and *gw*Cry1-PHR proteins were subcloned into a pVL1392-derived baculovirus transfervector to allow for subsequent recombinant expression of the target proteins in a *Sf9*/baculovirus expression system. For selective purification by means of immobilised metal affinity chromatography (IMAC) matrices, we expressed the genes as fusions with a vector-encoded N-terminal $(His)_6$-tag . The full length sequence of Cry1a (1866 bp) was subcloned into the polylinker region of the pDIA92B-His transfervector by *SmaI/NotI* digest. The PHR region of Cry1 (1536 bp) was subcloned into the polylinker region via *NcoI/NotI* digest. Recombinant baculoviruses were generated by cotransfection of transfer vectors and baculoviral genomic DNA and clonal isolation of Cry1 protein-expressing baculoviruses according to standard methods (Luckow & Summers 1988; Blissard & Rohrmann 1990).

Recombinant expression. To characterise the molecular properties of garden warbler Cry1a and Cry1-PHR proteins, we expressed the proteins in *Sf9* cells transfected with the corresponding recombinant baculovirus. Infected cells were incubated in ExCell420 culture medium (JRH) supplemented with 25 µM each of FAD and FMN (Sigma) in spinner flasks (0.6 l culture volume) for 66 h (± 4 h) at 27° C. Cell pellets were harvested, washed in PBS and quick frozen in liquid nitrogen.

Purification. *gw*Cry1a (628 aa; ~ 69 kDa) and *gw*Cry1-PHR (510 aa; ~ 56 kDa) were expressed as soluble fusion proteins and purified to near homogeneity by means of immobilised metal affinity chromatography (IMAC) in 20 mM HEPES buffer (pH 7.4) containing 500 mM NaCl and 50 µM FAD. For *gw*Cry1a an extra on-column denaturation/refolding procedure was added to the purification protocol as the C-terminal extension seems to prevent correct binding of the flavin cofactor. Denaturing and refolding the protein while fixed to the column allows for controlled conditions during the renaturing step; the bound protein is sterically isolated from other protein molecules, which prevents aggregation of folding intermediates and losses from precipitated protein. Recombinant

*gw*Cry1a was bound to the Chelating Sepharose matrix and unspecifically bound contaminants were washed away with 20 mM HEPES buffer containing 500 mM NaCl and 50 µM FAD (pH 7.4). The column-bound protein was denatured by a linear gradient (10 column volumes) of 0 M to 6 M urea in 20 mM HEPES buffer containing 50 µM FAD, renatured by a "reversed" linear gradient from 6 M to 0 M urea in 20 mM HEPES 50 µM FAD buffer, and finally eluted by increasing imidazole concentration. Samples were concentrated in Vivaspin contractor columns (membrane: 30.000 MWCO) (Sartorius). Excess unbound flavin was eliminated by a final desalting step using HiTrap 5 ml columns (Amersham Bioscience). Samples were stored in 20 % glycerol at a final protein concentration of 15-25 µM, shock frozen in liquid nitrogen and stored at -80° C.

The quality of the purified proteins was analysed by SDS-PAGE and Western blot analyses using standard procedures. On PVDF membrane (Pall) Western blots *gw*Cry1a protein was probed by goat polyclonal antibody α(anti)-Cry1 (A-20) *sc-5953* (Santa Cruz Biotechnoloy), and Cry1-PHR was detected by a commercially available mouse monoclonal α-His tag antibody (PentaHis antibody, Qiagen); the primary antibodies were detected by appropriate alkaline phosphatase-conjugated secondary antibodies and nitroblue tetrazolium/bromo-chloro-indoxyl phosphate staining on the membrane.

Uv/vis spectral analysis Purified *gw*Cry1a and its N-terminal PHR domain from the migratory garden warbler, containing FAD in its fully oxidised redox state, were supplemented with 5 mM DTT, deoxygenated and illuminated at 290 K with blue light (Halolux 30HL, Streppel, Wermelskirchen-Tente, Germany) selected with a 420-470 nm band filter (Schott, Mainz). Reoxidation was performed under aerobic conditions in the dark. The concentration of FAD was estimated based on its absorbance at 450 nm (ε_{580} = 11.3 × 10^4 M^{-1} cm^{-1}). The concentration of the holoprotein (apoprotein with its flavin cofactor built in) of the *gw*Cry1-PHR construct was calculated to be 9 µM, the concentration of the *gw*Cry1a holoprotein was calculated to be 3.5 µM.

Transient Absorption experiments (TA) Solutions of proteins (concentrations varied between 15-25 µM) in 20 mM HEPES buffer and 20 % glycerol at 270 K were photoexcited at 355 nm using a Nd-YAG laser (5 ns, 5 mJ/pulse). The monitoring beam was provided by a Tungsten lamp bulb (150 W). The light from the sample was fed to a Monochromator Newport model 77250 before entering a photomultiplier tube Hamamatsu R-928. The time resolution of

the measurement was 20 μs. The time traces obtained in Figure 4 represent the average obtained from 2 signals.

Results

We successfully expressed *gw*Cry1a and *gw*Cry1-PHR as soluble flavoproteins, containing FAD in fully oxidised form (exemplarily shown for *gw*Cry1a in Fig.1). Absorption spectra resembling that of flavoproteins were measured for purified *gw*Cry1a and *gw*Cry1-PHR proteins. FAD cofactors show characteristic optical absorption properties in all three biologically relevant redox states: fully reduced ($FADH^-$ or FADH2), semiquinone radical ($FAD^{\bullet -}$ or $FADH^{\bullet}$), and fully oxidised (FADox). All cryptochrome constructs were isolated in a yellow form characteristic of a flavoprotein with a fully oxidised FADox. The protein has absorption maxima at 450 and 366 nm representing the S0 $\rightarrow$ S1 and S0 $\rightarrow$ S2 transitions of its flavin cofactor, respectively (see Fig. 2). Continuous illumination of the sample diminished the intensity of these bands (but recovered by reoxidation). In contrast to other characterised members of the photolyase/cryptochrome superfamily, no well-resolved vibration side bands at 430 and 470 nm are observed. These are normally indicative of tight and well-defined non-covalent binding of the chromophore to the highly ordered protein structure. After illumination with blue light ($420 < \lambda < 470$ nm) in the presence of DTT as an exogenous electron donor, the formation of the fully (two-electron) reduced $FADH^-$ form of the flavin chromophore is observed, which has a broad spectral contribution at around 360 nm (Fig. 2). Futhermore, in contrast to all other members of the photolyase/cryptochrome superfamily, flavin radicals (exhibiting absorption maxima at 590 and 635 nm) are not generated to a significant amount. No significant radical formation is observed while reoxidising the proteins under aerobic conditions. These results clearly show that the FAD cofactor is protein-bound, because free FAD cannot be transformed into the fully reduced form under these experimental conditions.

Figure 3 shows the transient absorption spectra obtained for Cry1a as a function of both the time delay between laser flash and monitoring window (t_{delay} = 0.5, 3, 5, 9, 13 ms; width of time window is 500 μs) and wavelength of the monitoring beam (horizontal axis). Clearly, the spectra are strongly dependent on both parameters with the graph at early times (corresponding to a t_{delay} = 0.5 ms) exhibiting a pronounced absorption peak around 510 nm. As obvious from comparison of the five spectra, this peak has a much shorter life-time than the features at higher wavelengths. Indeed, Figure 4 shows this behaviour in a more quantitative fashion.

Here, the absorption of the sample is detected as a function of time after the laser flash for two wavelength regions (red curve: 490-550 nm; blue curve: 550-630 nm). The decay shown in the red graph can only be modelled satisfactorily by a double exponential decay with lifetimes of 4 ms and 14 ms, respectively whilst the longer-lived blue decay curve can be modelled very well using just a single exponential with life time 14 ms.

Discussion

In this study, we experimentally verified several key assumptions crucial for the theoretical concept of light-mediated magnetoreception in birds (Ritz *et al.* 2000). We succeeded in expressing Cry1a, which occurs in the retina of magnetically orienting night-migratory garden warblers (Mouritsen *et al.* 2004) and its highly conserved N-terminal PHR domain as holoproteins (with their flavin cofactor bound). The absorption spectrum of flavoproteins is dependent on the redox status of the bound flavin cofactor, which in turn is influenced both by the apoprotein the cofactor is bound to (in our case *gw*Cry1a and *gw*Cry1-PHR) and by the redox environment (Jorns *et al.* 1984). Garden warbler Cry1a and Cry1-PHR purified from insect cells both contained the flavin cofactor in the fully oxidised state (Fig. 2), as has also been shown for isolated *At*cry1 from *A. thaliana* (Lin *et al.* 1995). In isolated photolyases, however, the flavin cofactor is usually bound in a less oxidised state (e.g. Sancar 1994; Yasui *et al.* 1994). The absorption spectrum of garden warbler Cry1a fits well with the fact that songbirds, including garden warblers, are well-oriented using only their magnetic compass under blue and turquoise light, whereas they seem challenged performing magnetic compass orientation under yellow and red light (Wiltschko *et al*. 1993, 2004; Wiltschko & Wiltschko 1995). The fact that songbirds are well-oriented under green light does not fit well with the absorption spectra of the fully oxidised form of garden warbler cryptochrome. However, under natural conditions, a significant part of the *gw*Cry1a may exist in the semiquinone form, which is expected to absorb in the spectral wavelengths-range green to yellow (Lin *et al.*1995). This might explain the magnetic orientation capabilities of songbirds under green light and their apparent abilities to adapt to orienting magnetically under light of long wavelengths. Alternatively, a second detector could be involved (e.g. Wiltschko *et al.* 2004).

Using a laser flash photolysis experiment, we were able to prove that illumination (at 355 nm) of Cry1a of the migratory garden warbler produces radical pair species with life times of the order of milliseconds. This is a crucial finding as the evolution of a magnetic field effect relies

on the existence of radical pairs that are long-enough lived for the field to have sufficient time to affect the singlet-triplet mixing processes in the pair. For a field as weak as that of the Earth, the life time should exceed at least a microsecond, a condition clearly fulfilled by the radical species investigated here.

For a discussion of our Transient Absorption results we turn to a recent paper by Giovani *et al.* (2003) who proposed a reaction scheme for the *A. thaliana* plant Cry1 (*At*cry1): illumination of the sample by the laser pulse is followed by rapid formation of the flavin radical (the semiquinone or semireduced form) and a tryptophanyl radical. This process is followed by an electron transfer from a tyrosine to a tryptophanyl radical (a process on a 1 ms time scale), which is accompanied by a pronounced positive contribution to the TA spectrum in the 460–560 nm range. Furthermore, Giovani *et al.*(2003) observes two slow components (5 ms and 100 ms, respectively) which closely follow the FADH•-FAD difference spectrum and correspond to the FADH•-TryO• back electron transfer. We hence believe strongly that our *gw*Cry1a sample shows very similar behaviour to the *At*cry1. The two life times reflecting the Tyr-Trp• electron transfer (4 ms) and the FADH•-TryO• back electron transfer (14 ms) mirror those observed for *At*cry1 (1 ms and 5 ms, respectively) taking into account not only the different nature of the two cryptochromes, and hence the environment of the cofactors in these systems, but also the fact that we worked at 270 K (cf. 285 K in Giovani's work). The pronounced feature at 510 nm for TA measurements at short t_{delay} (red curve in Fig. 3) is most likely attributable to the Tyr-Trp• electron transfer (again, in accordance with Giovani's findings).

Sample degradation is an important factor affecting the TA experiments. It was observed that the TA signal changed with the number of laser shots the sample had been subjected to. This was mainly reflected in a decreased (ground state) bleaching signal at wavelengths below 490 nm which we tentatively subscribe to the formation of fully reduced flavin. Blowing Oxygen over the sample for approximately 2 min rapidly returned the sample to its original state (as determined by TA measurements).

The long life time (> 1 ms) of Cry1a from migratory garden warblers as well as *gw*Cry1-PHR, the photolyase homology region, further strengthens their suggested role as primary magnetic compass receptor molecule, because the sensitivity of the radical-pair system to weak magnetic fields increases significantly with longer life times of the radical species (Ritz *et al.* 2000).

Figure legends

Figure 1 SDS-PAGE of recombinantly expressed garden warbler cryptochrome (exemplarily shown for *gw*Cry1a protein). **A** Coomassie stained SDS gel, and **B** Western-Blot (with α-Cry1 antiserum from immunised goat), both showing identical fractions of the IMAC purification process. *gw*Cry1a (628 aa) is recognisable as a distinct band at ~ 62 kDa in both the coomassie stained SDS gel and on the Western-blot (indicated by the arrowhead to the right). *1 gw*Cry1a lysat (protein extract from insect cell pellet, 1:10 dilution); *2 gw*Cry1a cleared lysat (centrifuged and filtered lysat, 1:10 dilution); *3 gw*Cry1a flow through (after sample loading on column, 1:10 dilution); *4 gw*Cry1a elution step1 (e1) [50% Imidazol]; *5 gw*Cry1a elution step2 (e2) [100% Imidazol] protein quantity for e2 is 5 µg; *6* negative control (recombinantly expressed human La/SSB autoantigen; ~ 48 kDa) confirms the specificity of the antibody.

Figure 2 Optical spectroscopy. UV/vis absorption spectrum of **A** *gw*Cry1-PHR [9 µM]and **B** *gw*Cry1a holoprotein [3.5 µM]from migratory garden warblers, recombinantly expressed in baculovirus-infected *Sf9* insect cells. Both samples were illuminated under anaerobic conditions with blue-light for the times indicated (the quantitative difference in absorption is due to concentration-differences). Reoxidation was achieved by bubbling air into the optical cuvette. The dark spectra (black) reflect the absorption curve of the fully oxidised form of *gw*Cry1a and *gw*Cry1-PHR. After beaming blue light for 330 seconds on *gw*Cry1a and for 540 seconds on *gw*Cry1-PHR, the cryptochromes are converted into the fully reduced form, which is reflected in the spectra for these time points. After 2 respectively 3 minutes of reoxidation the cryptochromes are back in their fully reduced form as seen by the fact that these spectra are identical to the original dark spectra. Under the conditions used here, no semiquinone-intermediate forms were observed.

Figure 3 Transient absorption spectra for *gw*Cry1a as a function of wavelength (monitoring beam). The five graphs reflect the optical properties of the sample at different times, t_{delay}, after the laser flash. The spectra were obtained by averaging (two) time profiles (cf. Fig. 4) centred at t_{delay} using a time window of 500 µs. For a detailed analysis of the spectra, see main text.

Figure 4 Transient absorption time profiles for *gw*Cry1a of the migratory garden warbler. A laser flash (indicated by the arrow) is given at time 0. The transient signal is obtained by averaging 7 time traces for the 490-550 nm wavelength region in 10 nm steps (blue curve, left scale) and 9 time traces for the 550-630 nm region in 10 nm steps (red, graph, right scale),

respectively. Solid lines show the fitted exponential decays. The laser flash leads to radical formation as reflected by an absorption value above 0. As long as the absorption curves are significantly above zero, there are radicals produced by the laser flash left, i.e. for ~ 25 ms. The chemically well-defined term, "life-time" of the radicals, can be deduced from these curves by fitting first or second order exponentials. These calculations revealed a "life-time" of ~ 14 ms for Cry1a of migratory garden warblers. For further discussion, see main text.

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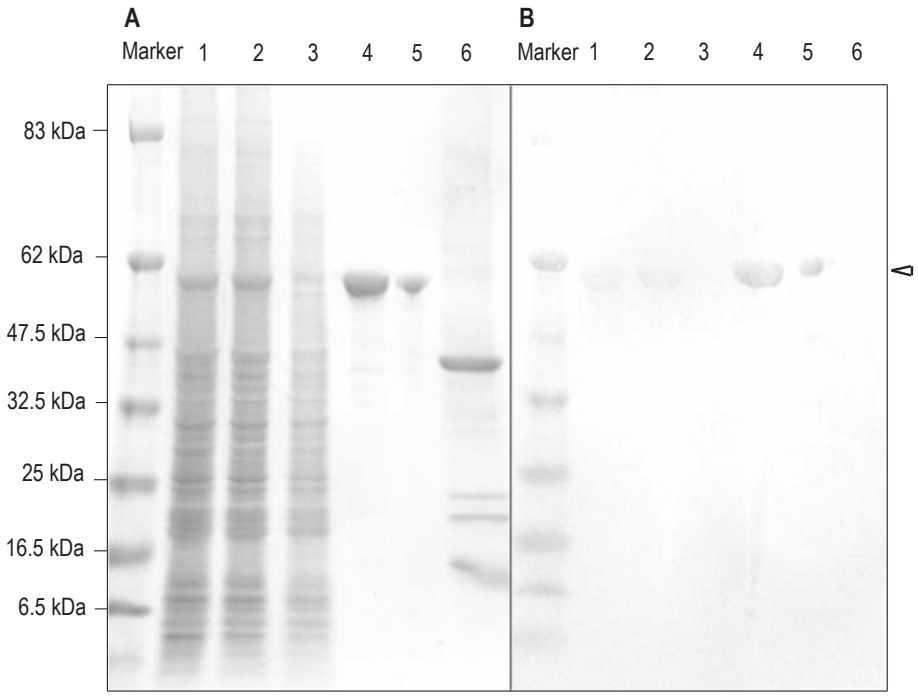
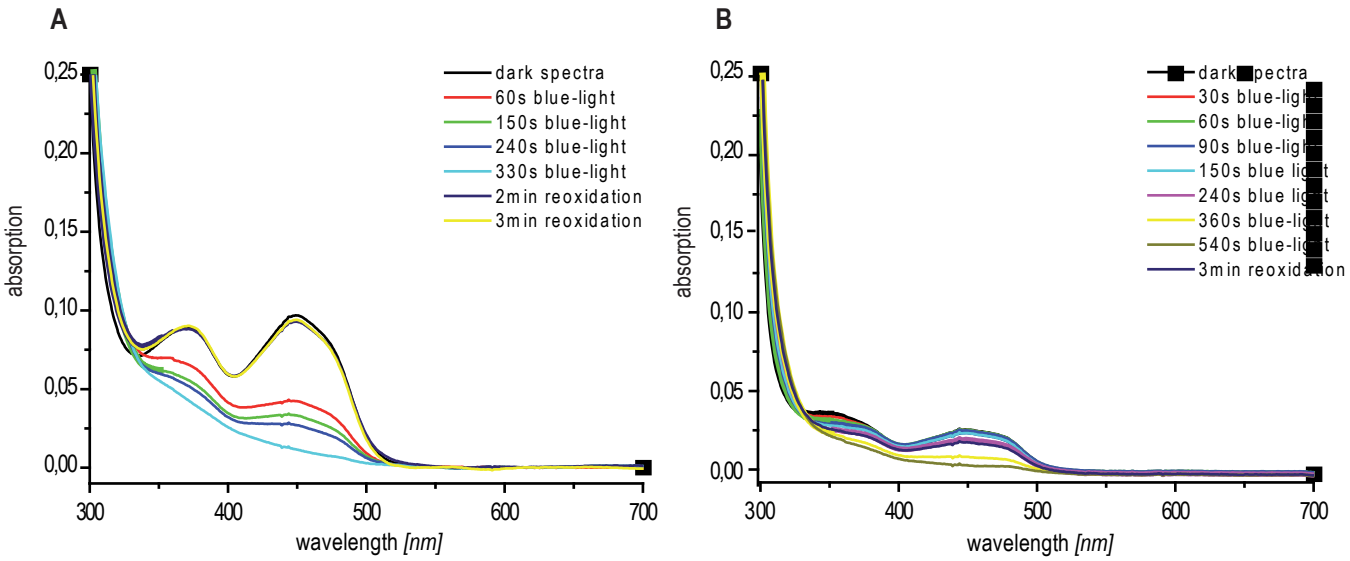

A
Marker 1 2 3 4 5 6
B
Marker 1 2 3 4 5 6
83 kDa
62 kDa
47.5 kDa
32.5 kDa
25 kDa
16.5 kDa
6.5 kDa

Figure 1


A
absorption
wavelength [nm]
dark spectra
60s blue-light
150s blue-light
240s blue-light
330s blue-light
2min reoxidation
3min reoxidation
B
absorption
wavelength [nm]
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540s blue-light
3min reoxidation

Figure 2

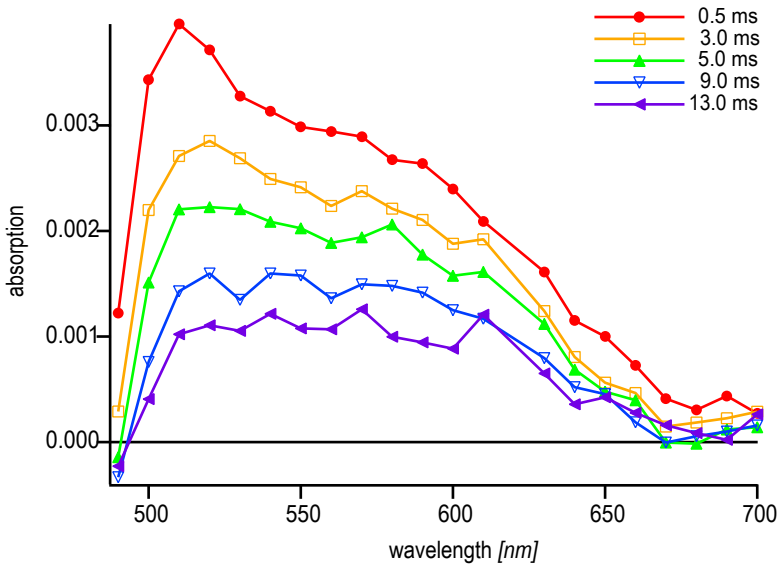

0.5 ms
3.0 ms
5.0 ms
9.0 ms
13.0 ms
absorption
0.003
0.002
0.001
0.000
500
550
600
650
700
wavelength [nm]

Figure 3

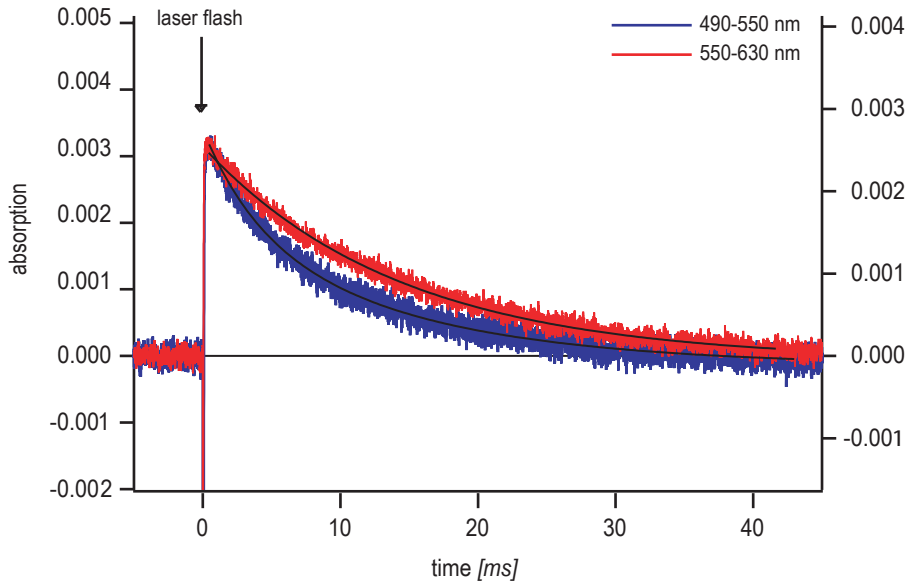

laser flash
490-550 nm
550-630 nm
absorption
0.005
0.004
0.003
0.002
0.001
0.000
-0.001
-0.002
0.004
0.003
0.002
0.001
0.000
-0.001
0
10
20
30
40
time [ms]

Figure 4

*Supplementary material **A***

Methods: Identification, cloning and recombinant expression of garden warbler cryptochromes.

Supplementary material **A: Identification, cloning and recombinant expression of garden warbler cryptochromes**

I. **cDNA synthesis of total RNA** from garden warbler (*Sylvia borin*) retina

RNA isolation was performed, using the NucleoSpin RNAII (Macherey-Nagel) kit, which includes treatment with DNase I to exclude contamination with genomic DNA. The concentration of isolated RNA was 700 ng/µl for the right eye and 38 ng/µl for the left eye. Because Wiltschko *et al.* (2002) suggested a marked dominance of the right eye/left brain hemisphere in the process of magnetoperception for magnetic compass orientation, all further proceedings are based on total RNA extraction from the right eye if not explicitly noted otherwise (transcripts were confirmed to be present in total RNA extracts of the left eye as well).

cDNA-synthesis was carried out using 450 ng RNA with Revert Aid^{TM} H Minus First Strand cDNA Synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) following the manufacturer's protocol. Hot start PCR amplification reactions were performed in 25 µl total reaction volume containing 1 µl of the cDNA synthesis reaction as template, 1x reaction buffer (SAWADY/Amersham-Pharmacia, depending on DNA polymerase used), 0.25 mM dNTPs (Pharmacia Biotech), of each primer 1.25 pmol/µl PCR volume, 1 unit of *Pwo* polymerase (SAWADY) or 2 units of *Taq* polymerase (Amersham-Pharmacia). The PCR reaction was carried out in a MWG Primus 25 thermocycler. The temperature profile for all hot-start PCR reactions was as follows: first denaturation 3 min 94° C; 45 cycles of 45 s at 94° C, 45 s annealing at 54° C and 40 s extension at 72° C (duration of extension phase depends on the expected size of the amplified fragment and enzyme; extension step comprised 1 min/kb DNA for amplification with *Taq* polymerase and 2 min/kb DNA for *Pwo* amplification); the last cycle was extended to 5 min at 72° C to ensure completion of the final extension. cDNA synthesis and contamination with genomic DNA was controlled by using intron-spanning β-actin-specific primers [$p673^{OL}$ ($USP^{\beta\text{-}actin2}$) 5'-GGCATGTGCAAGGCCGGCTTC-3' (exon 2); and $p674^{OL}$ ($DSP^{\beta\text{-}actin4}$) 5'-GGATGGCATGAGGGAGCGCGT-3' (exon 4)[1]] with the following conditions: 2 min at 95° C; 35 cycles of 30 s at 95° C, 1 min at 64° C, and 1.5 min at 72° C; and 15 min at 72° C).

[1] All primers used in this study (primer number, - name and oligonucleotide sequence), are summarised and clearly laid out in *Suppl. I* Table 1 of Appendix. I.

II. **Cloning strategies**

II. 1. Cloning strategy for ***gw*Cry1a** [GenBank accession no. AJ632120]

Full length sequence information of *gw*Cry1a (*gw* = garden warbler) was derived by a number of steps. To establish an anchor of garden warbler specific sequence information, a pair of degenerate oligonucleotide primers was designed based on an alignment of known sequence information for cryptochrome 1 from chicken (*Gallus gallus*) [GenBank accession no. AY034432], man (*Homo sapiens*) [NM0049705], mouse (*Mus musculus*) [NM007771] in regions of high homology (*Supplementary* Figure 2, *Supplementary material B*). The oligonucleotides correspond to positions 1272-1287 [$p675^{OL}$, 5'-TTGTCGACAATGCTGGAA G(CT)TGGA-3', 5' end of primer has added *SalI* site for cloning] and 1896-1916 [$p676^{OL}$, 5'-TTGAATTCTTCTTC(CT)(GT)GA(CT)T(AT)GG(AG)CG-3', 3' end of primer has added *EcoRI* site for cloning] of the chicken Cry1 cDNA-sequence AY0304432. Based on the alignment, the expected PCR product size from a Cry1 cDNA of the different species mentioned above would vary between 539 and 644 bp.

PCR amplification with the degenerate primer pair resulted in a 642 bp Cry1a PCR product from both left and right garden warbler retina cDNA. The reaction was carried out with 1 unit of *Pwo* DNA polymerase (SAWADY), using the following cycle conditions: 3 min at 94° C; 40 cycles of 30 s at 94° C, 30 s at 59° C, and 1 min 20 s at 72° C; and 2 min at 72° C. The amplified fragment was restriction-digested with *SalI* + *EcoRI*, subcloned into the polylinker region of a pBC-SK(+) vector (Stratagene) and transformed into Ca^{2+}competent XL1-Blue MRF' cells. 2 independent clones were purified as plasmid DNA (named #731, #732) with a Plasmid Midi Preparation kit (Qiagen) and sequenced (external sequencing service provider Solvias, Basel; PCR cycle sequencing with fluorescent terminators and analysis on an ABI3730 automated sequencer). The resulting species-specific sequence information of garden warbler cryptochrome 1a is incorporated in *Supplementary* Figure 2 (*Supplementary material B*) and confirms a sequence previously determined for this Cry1a section by Petra Dirks (Mouritsen *et al.* 2004, Paper II).

Based on this internal 642 bp fragment sequence, primer sets were designed to PCR amplify 2 neighbouring fragments encoding a N- and C-terminal region of Cry1a, which were then used to reconstruct the full-length *gw*Cry1 coding sequence. PCR amplification (3 min at 94° C; and 40 cycles of 45 s at 94° C, 45 s at 54° C, and 2 min at 72° C) required an "long-distance PCR"

enzyme mix of 2 units *Taq* DNA polymerase (Amersham-Pharmacia) + 0.04 units *Pwo* DNA polymerase (SAWADY) (amplification with the preferable proofreading *Pwo* polymerase alone did not succeed, also not with various concentrations of PCR additives, e.g. dimethylsulfoxide (DMSO), betaine, glycerol).

For amplification of the N-terminal end (1477 bp) the upstream primer *p677*, 5'-AGCA CGGTCTCCCATGGGGGTGAACGCCGTGCACTGGTT-3', could be designed based on the absolute conservation of the first 30 Cry1-coding nucleotides between bird and mammalian species (see sequence alignment in *Supplementary* Figure 2, *Supplementary material B*); on the 5' side primer *p677* contains an added *BsaI* restriction site (all restriction sites incorporated in primer design are marked as underlined), which liberates a *NcoI*-compatible restriction overhang at the exact position of the Cry1 start codon. The downstream primer *p678*, 5'-AGCA AGGTCTCCTTTCATCCTCTCAATATTCAGA-3' was designed from the internal sequence of the 642 bp fragment; on its 5' side it contains an added *BsaI* site to generate a designed overhang for joining to the C-terminal Cry1 fragment. The PCR-amplified and blunt-ended fragment was subcloned into an *EcoRV* digested pT7Blue-3 vector using the Perfectly Blunt cloning kit (Novagen, cloning method based on "phosphorylated insert into dephosphorylated vector") and transformed into NovaBlue cells. A selected insert-containing clone (named #743) was prepared as plasmid DNA and sequenced (for details compare for *Supplementary* Figure 3, *Supplementary material B*).

The C-terminal fragment of garden warbler Cry1a (440 bp) was PCR amplified with the primer combination *p681*, 5'-AGCACGGTCTCCGAAACAGATCTACCAGCAGCTT-3' (sequence based on the 642 bp fragment; primer contains added *BsaI* site for joining to N-terminal fragment) and *p682* 5'-AGCAAGCGGCCGCTTATCAATTTGTGCTCTGCCGCTGGA CTTT-3' (sequence corresponds to the chicken Cry1a sequence, which is a reasonable choice because of the very high similarity of chicken, man and mouse Cry1 nucleotide sequences at the end of their protein coding regions; primer contains double stop codons for the Cry1a coding region and a *NotI* site for cloning). The amplified fragment was digested with *NotI* resulting in a 429 bp fragment, ligated (Rapid Ligation kit, MBI) as a blunt/*NotI* fragment into *SmaI/NotI* digested pBC-SK(+) vector, and transformed into Ca^{2+}competent XL1-Blue MRF' cells. Plasmid DNA of this clone (#745) was purified and sequenced as described above (for details see *Supplementary* Figure 4, *Supplementary material B*).

I assembled the complete coding region for garden warbler Cry1a protein by subcloning the N- and C-terminal cDNA parts together into a pVL1392-derived baculovirus transfer vector, which allows for subsequent recombinant expression of the target proteins in a *Sf9*/baculovirus expression system. The N-terminal part was isolated from plasmid #743 as a *SmaI*/*BsaI* DNA-fragment, the C-terminal part from plasmid #745 as *BsaI*/*NotI* fragment. In a 4 fragment-ligation both Cry1a parts were combined with *NcoI*→*Pwo* DNA Polymerase-filled/*ApaI* pDIA92B-His and *ApaI*/*NotI* pDIA92B transfer vector fragments: the resulting Cry1a transfer vector is numbered #752 (*Supplementary* Figure 5, *Supplementary material B*). This transfer vector provides a vector-encoded N-terminal $(His)_6$-tag, enabling the selective purification of a $(His)_6$-Cry1a fusion protein by means of immobilised metal affinity chromatography (IMAC) matrices. Generation of recombinant baculoviruses is described in Section V, below (cloning strategy for the full length *gw*Cry1a transfer-vector is illustrated in *Supplementary* Figure 5, *Supplementary material B*; sequence information and protein data of *gw*Cry1a are listed on subsequent pages).

II. 2. Cloning strategy for ***gw*Cry1b** [GenBank accession no. DQ838738]

The same procedures were applied to derive the full length sequence of garden warbler specific Cry1b. Primer combination used for PCR amplification of a C-terminal end (341 bp) of garden warbler Cry1b was *p681* (as above for Cry1a) and *p708*, 5'-AGCAAGCGGCCGCTTATCATT CTGATGTTTTGTCTGGTTT-3' (sequence is based on a back-translation of the European robin (*Erithacus rubecula*) *e*Cry1b C-terminus (*e* = European robin) (Möller *et al.* 2004); primer contains double stop codons for the Cry1b coding region and a *NotI* site for cloning). The amplified fragment (341 bp) was cloned into the blunt vector pCR4BLUNT-TOPO (GeneRacer™ Kit, Invitrogen), yielding the intermediate plasmid #780 (for cloning strategy see *Supplementary* Figure 6, *Supplementary material B*). The sequence-verified garden warbler Cry1b C-terminal was cloned together with the Cry1 N-terminal fragment into a baculovirus transfer vector exactly as described above for Cry1a resulting in the product clone named #800 (chosen from two identical sister clones) (exact cloning strategy illustrated in *Supplementary* Figure 7, *Supplementary material B*).

II. 3. Cloning strategy for ***gw*Cry1-PHR**

A recombinant protein containing amino acid residues 1 to 509 of garden warbler Cry1 was chosen for expression of the Cry1 photolyase homology region (PHR); design considerations are based on the known crystal structure of *Arabidopsis thaliana* Cry1-PHR and the point of C-

terminal sequence divergence between gwCry1a and gwCry1b (details discussed in section *II.3.1. Results I* of the *Introduction*, *pp. 41-42*). A PCR fragment representing the shortened C-terminal end of the photolyase homology domain (PHR) was obtained by amplifying a cloned gwCry1a-templates with the primer pair *p741*, 5'-TAGTTCCTTCTTTCAGCAGTTT-3'; and *p742*, 5'-AGCAAGCGGCCGCTTATCAAGTTGCAAGAAGACCCAGTCCT-3' (primer contains double stop codons for terminating the Cry1-PHR after aa 509, and a *NotI* site for cloning). The amplified 320 bp fragment was digested with *BstXI/NotI*, resulting in a 107 bp target fragment. This fragment was used to exchange the Cry1a C-terminus of transfer vector #752 against the PHR C-terminus by the cloning strategy shown in *Supplementary* Figure 8 (*Supplementary material B*) requiring a 1408 bp N-terminal *NcoI/BstXI* Cry1 fragment. From several identical sister clones, the transfer vector plasmid #876 was chosen for baculovirus expression of the N-terminally $(His)_6$-tagged Cry1-PHR.

Expression of a recombinant protein as a fusion to e.g. a His-tag sequence carries the potential danger that presence of the fusion partner disturbs folding or biological function of the target protein. As an alternative to the N-terminally $(His)_6$-tagged Cry1-PHR a version with the $(His)_6$-tag moved to the C-terminus was constructed. A His-tagged PHR C-terminal fragment was PCR amplified with primers *p741* (see above) and *p743*, 5'- AGCAAGCGGCCGC TTATCAATGGTGATGGTGATGGTGAGTTGCAAGAAGACCCAGTCCT -3' (primer contains a $(His)_6$-tag after aa 509, double stop codons and a *NotI* site for cloning). Cloning was carried out by a strategy similar to the previous PHR construct and resulted in plasmid #879 (data for exact cloning strategy of the C-terminally tagged construct not shown; for general sequence information of *gw*Cry1-PHR see *Supplementary* Figure 8 and subsequent sequence data, *Supplementary material B*).

The baculovirus/*Sf9* insect cell system is the main expression system used for this work. As a potential alternative, *Escherichia coli (E. coli)* – based bacterial expression with the T7 RNA polymerase/pET system (Studier *et al.* 1990) was tried for the Cry1-PHR. N- and C-terminally $(His)_6$-tagged versions of the Cry1-PHR region were subcloned into the *NcoI/NotI*–opened polylinker region of pET24d(+) (exact cloning strategy for the *E. coli* expression vectors not shown, for general sequence information of the *gw*Cry1-PHR sequence see *Supplementary* Figure 8 and subsequent sequence data, *Supplementary material B*). Expression tests of these plasmids in *E. coli* strains BL21(DE3) and Tuner(DE3) by standard methods did not show detectable accumulation of recombinant Cry1-PHR. Therefore, this approach was not followed further.

II. 4. Cloning strategy for partial coding sequences of ***gw*Cry2** [GenBank accession no. AY739908] and ***gw*Cry4**

For detection of expression of cryptochrome 2 in garden warbler retina I used a similar strategy as for the first garden warbler specific Cry1a transcript. PCR amplification of garden warbler retina cDNA was carried out with the primer pair *p680*, 5'-ACGAAGAATTCGACGAAAGC CACATCCAG-3';and *p679*, 5'-AGCACGTCGACGAACCCCATCTGCATCCA-3' (primer sequences derived from a sequence alignment of cryptochrome 2 cDNAs from mouse [NM009963], man [NM021117], chicken [AY034433]; primers hybridise at strongly conserved sections of the Cry2 cDNAs). The resulting approx. 240 bp long PCR product was cloned into pTBlue-3 by means of the Perfectly Blunt cloning kit (Novagen) and transformed into NovaBlue cells. Plasmid purification and sequencing was carried out as described for fragments of *gw*Cry1a.

Initial sequence analysis of the plasmid clones revealed that the majority of clones contained an insert which corresponded to a Cry4 transcript, based on similarity to the known Cry4 cDNA sequences from chicken [AY102068] and zebrafish (*Danio rerio*) [NM131787]. A representative clone of the Cry4 PCR product is plasmid #739 (*Supplementary* Figure 11, *Supplementary material B*). The amplification of Cry4 sequences can be explained by cross-priming of oligonucleotides *p679* and *p680* at sequences conserved between Cry 2 and Cry 4.

The search of a clone containing a Cry2 PCR product was carried out with a screen for internal *NcoI* restriction sites: the garden warbler Cry4 insert of plasmid #739 carries an internal *NcoI* site, whereas the chicken Cry2 cDNA lacks an *NcoI* site at the corresponding position. In the hope that garden warbler Cry2 can be identified by a similar lack of an internal *NcoI* site, 12 subclones carrying the PCR insert were digested analytically with *NcoI*: a single clone, plasmid #748, was not susceptible to *NcoI* digest and on sequence analysis was found to carry a 237 bp Cry2 cDNA sequence with 92 % homology to *Gallus gallus* Cryptochrome 2 mRNA [AY034433].

Based on the internal Cry2 sequence from clone #748, a 777 bp 3'-part of garden warbler Cry2 cDNA was amplified in a nested PCR using the following primer pairs: *p697*, 5'-TTATGAGGCCCCCGATTTTCTGTTG-3'; *p693*, 5'-AATCCCCTGAGACAGGAACCCTG AAGCC-3'; subsequent nested PCR: *p698* 5'-GTTCCTGCTCCTCTGGTCACCTCTT-3'; *p694*, 5'-AGGAACCCTGAAGCCTTGGCAAAG-3'. The amplified fragment was cloned into

pCR4Blunt-TOPO; plasmid #762 (for cloning strategy of *gw*Cry2 see *Supplementary* Figures 9 & 10; for *gw*Cry4 see *Supplementary* Figure 11, *Supplementary material B*). Immunohistological data show that *gw*Cry2 is localised in the nucleus, whereas *gw*Cry1a showed cytosolic localisation (Mouritsen *et al.* 2004, Paper II). Any cryptochrome working as a primary magnetoreceptor has to be oriented in the cell (e.g. linked to cytosolic skeleton proteins); therefore, a nuclear localisation of any candidate molecule suspected to be involved in magnetoperception is less likely. Furthermore, cryptochromes, located in the nucleus are likely to be involved in regulation of the circadian clock. For these reasons, I concentrated on the expression and purification of *gw*Cry1a and 1b during the time of my Ph.D. project. In a future extension of this project, the methodological approach I have chosen for recombinant expression of *gw*Cry1a, 1b and 1-PHR will be carried forward for full length cloning and subsequent recombinant expression of garden warbler Cry2 and Cry4.

III. **Homology and similarity scores**

All cloned fragments were subjected to nucleotide sequencing, and DNA similarities and identity scores were analysed by FASTA and WU-BLAST2 algorithms.

Garden warbler Cry1a (1863 bp) and Cry1b (1767 bp) have complete sequence identity in base pairs 1-1689. The C-terminal extensions of Cry1a (174 bp/58 aa) and Cry1b (107 bp/35 aa) show no sequence homology. *gw*CRY1a [AJ632120] full length nucleotide sequence WU-BLAST2 data base search reveals 96 % homology (96.618 % identity in FASTA search) with European robin *e*Cry1a [AY585716] and 93% homology (93.783 % identity by FASTA search) with chicken (*Gallus gallus*) Cry1 [AY034432]. *gw*CRY1a and *e*CRY1a are homologous to Cry1 from domestic chicken (Haque *et al*. 2002).

Comparison of *gw*CRY1b and *e*CRY1b by WU-BLAST2 and FASTA algorithms reveal 96 % sequence homology. *gw*CRY1b differs from *gw*Cry1a by its C-terminal extension. In European robins, the different C-termini of *e*CRY1a and *e*CRY1b sequences have been shown to be encoded in different exons (Möller *et al.* 2004). For chicken, no alternative splicing products have been reported so far. However, having the full chicken genome sequences available, a closer look at the genomic loci, its homologies to the *Sylvia borin* and *Erithacus rubecula* Cry1a/b cDNA isoforms, and donor/acceptor site predictions (using WISE2) suggests similar splicing variants in chicken as found in garden warbler and European robin (preliminary analysis of the genomic sequence of *Gallus gallus* chromosome 1).

Data base comparisons by WU-BLAST2 and FASTA algorithms for the 3' partial coding sequence of garden warbler Cry2 (plasmid #762) reveal 89 % sequence homology with chicken Cry2 [AY034433].

Data base search with WU-BLAST2 and FASTA algorithms for the partial coding sequence (plasmid #739) of *gw*Cry4 reveals 89 % sequence identity score with chicken Cry4 [AY102068] and 95 % identity with House sparrow (*Passer domesticus*) cryptochrome 4 [AY494987].

IV. **Recombinant expression** of *gw*Cry1a, *gw*Cry1b, *gw*Cry1-PHR in the baculovirus/*Sf9* expression system

To characterise the molecular properties of garden warbler Cry1a, Cry1b and Cry1 photolyase homology region proteins, I expressed and purified recombinant forms of the proteins from *Sf9* (*Spodoptera frugiperda*) insect cells infected with the corresponding recombinant baculovirus (Luckow & Summers 1988; Blissard & Rohrmann 1990; for a recent review see Kost *et al.* 2005). This expression system is based on the very strong polyhedrin promoter in the genome of the *Autographa californica* Nuclear Polyhedrosis Virus (in short, AcNPV baculovirus; a virus with circular double-stranded DNA genome). Since the polyhedrin gene is not essential for the virus life cycle in tissue culture, the structural gene encoding the polyhedrin protein can be replaced by heterologous cDNAs such as the Cry1 coding regions constructed in this work: between 2–3 days after infection of a susceptible *Sf9* insect cell with a recombinant baculovirus the transcriptional activity of the strong polyhedrin promoter leads to significant expression and accumulation of a recombinant target protein. A heterologous cDNA is moved into the baculovirus genome by co-transfection/lipofection of the intermediate transfer vector/cDNA and pre-linearised wild-type AcNPV DNA into *Sf9* cells: homologous recombination at homology regions, which flank the polyhedrin promoter/heterologous cDNA in the transfer vector, move the cDNA into the baculovirus genome. As this also recircularises the baculovirus genome, infectious recombinant virus particles can be produced by the insect cells. Single clonal recombinant viruses are isolated from culture supernatants of lipofected cells by plaque assay, a procedure which also allows to determine the viral titer (expressed in plaque-forming units per ml = pfu/ml) so that known amounts of virus can be used to infect cells during subsequent experimental work. Cell monolayers are infected with different dilutions (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) of virus. An overlay of agarose keeps the cells stable and limits the

spread of virus. When each infected cell produces virus and eventually lyses, only the immediate neighbouring cells become infected. Each group of infected cells is referred to as a plaque containing the clonal progeny of a single starting virus, and the plaques of lysed infected cells surrounded by the cell layer of uninfected cells can be visualised by light microscopy. Individual plaques obtained from varying dilutions of a viral stock can be counted to determine the viral titer (pfu/ml) of a given transfection or virus amplification supernatant. For the lipofection supernatants of the recombinant Cry1 constructs, I determined the following titers: $1.6 \cdot 10^8$ pfu/ml for *gw*Cry1a, $0.5 \cdot 10^8$ pfu/ml for *gw*Cry1b and $2 \cdot 10^8$ pfu/ml for *gw*Cry1-PHR (N-terminal $(His)_6$-tag) and $1 \cdot 10^7$ pfu/ml *gw*Cry1-PHR (C-terminal $(His)_6$-tag).

Well-separated plaques were picked for further amplification of clonal viruses, first by infecting *Sf9* cells in a well of a 6-well plate for 6 days, and then infecting *Sf9* cells in a 10 cm diameter petri dish with the 6-well supernatant for 3 days; the petri dish supernatant is designated Virus Stock I. The cell pellets from the petri dish were tested for expression of the recombinant Cry1 proteins by SDS-PAGE and Western blot analyses; also, preparation of detergent lysates and fractionation into insoluble and soluble lysate components allowed a rough evaluation of the recombinant protein's folding status (soluble protein typically is properly folded and native, whereas protein fractionating with in insoluble components typically is non-native and misfolded/denatured). On PVDF membrane (Pall) Western blots *gw*Cry1a protein was detected by goat polyclonal antibody α(anti)-Cry1 (A-20) *sc-5953* (Santa Cruz Biotechnoloy), *gw*Cry1b by rabbit polyclonal antibody raised against a peptide mapping at the carboxy terminus of Cry1b of migratory songbirds (European robin and garden warbler), and Cry1-PHR was detected by a commercially available mouse monoclonal α-His tag antibody (PentaHis antibody, Qiagen); the primary antibodies were detected by appropriate alkaline phosphatase-conjugated secondary antibodies and nitroblue tetrazolium/bromo-chloro-indoxyl phosphate staining on the membrane.

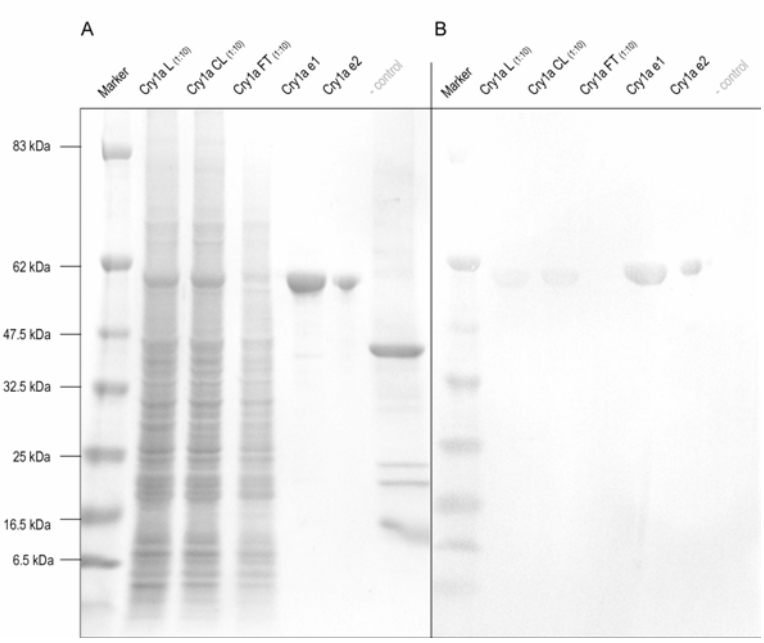
Of 5 tested clonal baculoviruses, 5 out of 5 were positive for expressions of *gw*Cry1a (and therefore represent a successful recombinant baculovirus); 4 out of 5 were positive for expression of *gw*Cry1b, and 5 out of 5 clonal baculoviruses (for both C- and N-terminally His-tagged) were positive for expression of *gw*Cry1-PHR. One selected recombinant baculovirus for each Cry1 construct was amplified by infecting 8 petri dishes with aliquots of Virus Stock I; the resulting Virus Master Stock was titered by plaque assay and can be stored long-term and used for the following preparative protein expression.

Low MOI (multiplicity of infection) virus stocks for large-scale protein expression work were prepared by infecting *Sf9* cells at a low MOI (<1; multiplicity of infection = plaque-forming units/cell number; "submolar" infection results in several infection cycles in the low MOI culture and in higher overall virus amplification), followed by incubation in ExCell^{TM} 420 (JRH) medium spinner flask cultures (see below) and supernatant harvest 4–5 days post infection. For large-scale protein production, infected *Sf9* cells (average MOI = 6) were incubated in spinner flasks (2 l flask volume / 0.6 l culture volume) with ExCell^{TM} 420 culture medium (JRH) supplemented with 25 µM each of FAD and FMN (Sigma) for 66 h (±4 h) at 27° C. Cell pellets, containing the intracellular recombinant cryptochromes, were harvested, washed in PBS and quick frozen in liquid nitrogen. Frozen cell pellets were lysed in insect cell lysis buffer containing protease inhibitor and benzonase (Merck). Lysate was cleared by centrifugation (20 min at 4° C, 18300 rpm) and filtered through a 1 µm pore size filter (Pall).

V. **Purification**

*gw*Cry1a (628aa; ~69kDa), *gw*Cry1b (595aa; ~65kDa) and *gw*Cry1-PHR (510aa; ~56kDa) were expressed as soluble His-tagged fusion proteins and purified to near homogeneity by means of immobilised metal affinity chromatography (IMAC) in 20mM HEPES buffer containing 500mM NaCl and 50µM FAD (Sigma). The purification protocol is based on the affinity of the six histidine residues for divalent cations, Ni^{2+} in our case, loaded on a metal chelating chromatography matrix (Chelating Sepharose Fast Flow, Amersham-Pharmacia). The fusion proteins selectively bind to the gel matrix and can be eluted by gradually increasing imidazole concentration. For *gw*Cry1a and *gw*Cry1b, an extra on-column denaturation/refolding procedure was added in the purification protocol as the C-terminal extension of the proteins seems to prevent correct binding of the flavin cofactor. Denaturing and refolding the protein while fixed to the column allows for controlled conditions during the renaturing step; the bound protein is sterically isolated from other protein molecules, which prevents aggregation of folding intermediates and losses from precipitated protein. Recombinant *gw*Cry1a or *gw*Cry1b were bound to the Chelating Sepharose matrix and unspecifically bound contaminants were washed away with 20 mM HEPES buffer containing 500 mM NaCl and 50 µM FAD, pH 7.9. The column-bound protein was denatured by a linear gradient (10 column volumes) of 0 M to 6 M urea in 20 mM HEPES buffer containing 50 µM FAD, renatured by a "reversed" linear gradient from 6 M to 0 M urea in 20 mM HEPES 50 µM FAD buffer , and finally eluted by increasing imidazole concentration. Samples were

concentrated in Vivaspin contractor columns (membrane: 30.000 MWCO) (Sartorius). Excess unbound flavin was eliminated by a final desalting step using HiTrap 5 ml columns (Amersham Bioscience). Samples were stored in 20 % glycerol at a final protein concentration of 15-25 µM, quick frozen in liquid nitrogen and kept at -80° C.



Supplementary **Figure 1: SDS-PAGE of recombinantly expressed protein, exemplarily shown for *gw*Cry1a: A** Coomassie stained SDS gel, and **B** Western-Blot (with α-Cry1 antiserum from immunised goat), both showing identical fractions of the IMAC purification process. *gw*Cry1a (628 aa) is recognisable as a distinct band at ~ 62 kDa in both, the coomassie stained SDS gel and Western-blot.

Abbreviations: L = lysat (protein extract from insect cell pellet); *CL* = cleared lysat (centrifuged and filtered lysat); *FT* = flow through (after sample loading on column), *e1* and *e2* denote two elution steps; protein quantity for *e2* is 5 µg. '*–control'* = neg. control for α-reaction (recombinantly expressed human La/SSB autoantigen; ~48 kDa.

VI. **References**

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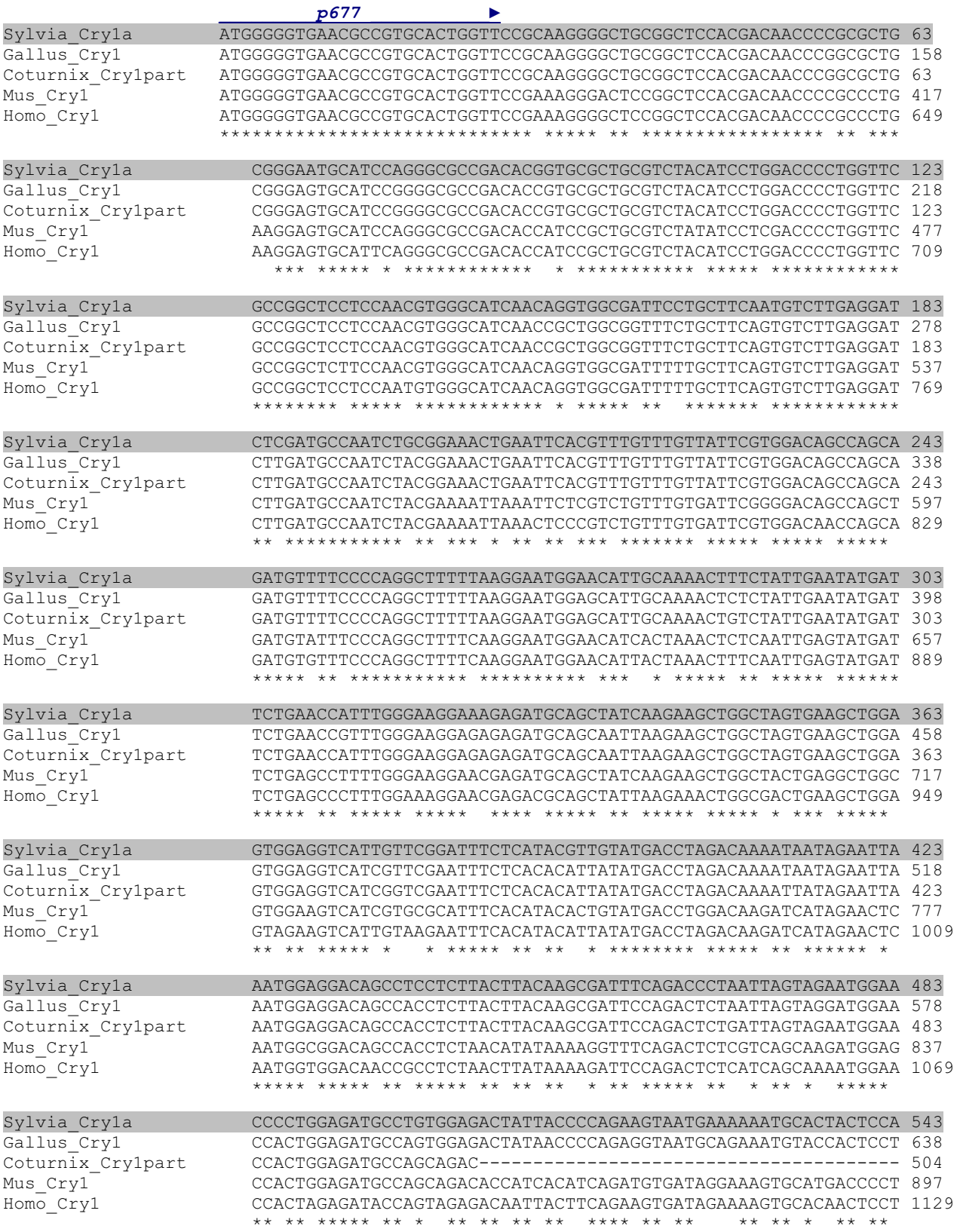
Supplementary material ***B***

Cloning strategies & sequence information: Cloning strategies and sequence data of garden warbler cryptochromes *gw*Cry1a, *gw*Cry1b, *gw*Cry1PHR and fragments of *gw*Cry2 & *gw*Cry4.

*Supplementary material **B***: **Cloning strategies & sequence information**

CLUSTAL W(1.83) Cry1(a) multiple sequence alignment and details of primer positions



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Sylvia_Cry1a            GTTTCTGATGATCACGATGAGAAATACGGTGTGCCATCCCTTGAAGAGCTGGGTTTTGAC 603
Gallus_Cry1             GTCTCTGATGACCATGATGAGAAATACGGTGTCCCGTCGCTTGAAGAGCTAGGTTTTGAC 698
Coturnix_Cry1part       ------------------------------------------------------------
Mus_Cry1                CTGTCTGATGACCATGATGAGAAATATGGCGTTCCTTCCCTGGAAGAGCTCGGCTTTGAT 957
Homo_Cry1               CTGTCTGATGACCATGATGAGAAATATGGAGTCCCTTCACTGGAAGAGCTAGGTTTTGAT 1189
                         * ******** ** ********** ** ** ** ** ** ******** ** *****

Sylvia_Cry1a            ACGGATGGTCTGCCTTCTGCAGTATGGCCAGGGGGAGAAACTGAAGCTCTCACACGATTA 663
Gallus_Cry1             ACAGATGGTCTGCCTTCTGCAGTATGGCCTGGGGGAGAAACAGAAGCTCTCACACGACTG 758
Coturnix_Cry1part       ------------------------------------------------------------
Mus_Cry1                ACAGATGGCCTGTCCTCTGCAGTGTGGCCAGGAGGAGAAACTGAGGCACTTACACGTTTG 1017
Homo_Cry1               ACAGATGGCTTATCCTCTGCAGTGTGGCCAGGCGGAGAAACTGAAGCACTTACTCGTTTG 1249
                        ** *****  *  * ******** ***** ** ******** ** ** ** ** **  *

Sylvia_Cry1a            GAAAGACATTTAGAACGAAAGGCTTGGGTAGCAAACTTTGAAAGACCACGAATGAATGCC 723
Gallus_Cry1             GAAAGGCATCTAGAACGAAAGGCTTGGGTAGCAAACTTTGAAAGACCACGAATGAACGCC 818
Coturnix_Cry1part       ------------------------------------------------------------
Mus_Cry1                GAAAGGCATTTGGAAAGAAAGGCCTGGGTGGCAAACTTTGAACGACCTCGAATGAATGCA 1077
Homo_Cry1               GAAAGGCATTTGGAAAGAAAAGCTTGGGTGGCAAATTTTGAAAGACCTCGAATGAATGCG 1309
                        ***** *** * *** **** ** ***** ***** ****** **** ******** **

Sylvia_Cry1a            AATTCCCTTCTGGCAAGCCCTACGGGGCTCAGCCCCTACCTCCGCTTTGGCTGTTTGTCC 783
Gallus_Cry1             AATTCCCTTCTGGCAAGCCCTACAGGGCTTAGTCCCTACCTCCGCTTTGGCTGTTTGTCC 878
Coturnix_Cry1part       ------------------------------------------------------------
Mus_Cry1                AACTCCCTGCTTGCAAGCCCAACTGGACTCAGTCCTTATCTCCGCTTTGGTTGTTTATCA 1137
Homo_Cry1               AATTCTCTGCTTGCAAGCCCTACTGGACTTAGTCCTTATCTCCGATTTGGTTGTTTGTCA 1369
                        ** ** ** ** ******** ** ** ** ** ** ** ***** ***** ***** **

Sylvia_Cry1a            TGTCGGCTCTTTTATTTCAAGTTAACGGATCTGTACAAAAAGGTAAAAAAGAACAGCTCC 843
Gallus_Cry1             TGTCGACTCTTTTATTTCAAGTTAACGGACCTGTACAAAAAGGTAAAAAAGAACAGCTCC 938
Coturnix_Cry1part       ------------------------------------------------------------
Mus_Cry1                TGTCGGCTGTTTTATTTCAAACTAACAGATCTCTACAAAAAGGTAAAGAAGAATAGTTCC 1197
Homo_Cry1               TGTCGACTGTTTTACTTCAAACTAACAGATCTCTACAAAAAGGTAAAGAAGAACAGTTCC 1429
                        ***** ** ***** *****  **** ** ** ************** ***** ** ***

Sylvia_Cry1a            CCTCCCCTCTCCCTCTATGGCCAGCTGTTATGGCGTGAATTTTTCTACACAGCGGCGACT 903
Gallus_Cry1             CCTCCCCTCTCCCTCTATGGCCAGCTGTTATGGCGTGAATTTTTCTACACAGCAGCAACT 998
Coturnix_Cry1part       ------------------------------------------------------------
Mus_Cry1                CCTCCCCTTTCTCTTTATGGGCAACTCCTGTGGCGTGAATTTTTTTATACAGCAGCCACA 1257
Homo_Cry1               CCTCCCCTTTCCCTTTATGGGCAACTGTTATGGCGTGAATTTTTCTATACAGCAGCAACA 1489
                        ******** ** ** ***** ** **  * ************** ** ***** ** **

Sylvia_Cry1a            AACAATCCACGGTTTGATAAAATGGAGGGGAATCCTATCTGTGTTCAAATTCCGTGGGAT 963
Gallus_Cry1             AACAATCCACGGTTTGATAAAATGGAGGGGAATCCTATCTGTGTTCAAATTCCATGGGAT 1058
Coturnix_Cry1part       ------------------------------------------------------------
Mus_Cry1                AACAACCCACGCTTTGACAAAATGGAAGGGAACCCCATCTGTGTTCAGATCCCTTGGGAC 1317
Homo_Cry1               AATAATCCACGCTTTGATAAAATGGAAGGAAACCCTATCTGTGTTCAGATTCCTTGGGAT 1549
                        ** ** ***** ***** ******** ** ** ** ********** ** ** *****

Sylvia_Cry1a            AAGAATCCTGAGGCTTTGGCCAAATGGGCAGAAGGCAGGACAGGTTTTCCTTGGATTGAT 1023
Gallus_Cry1             AAGAATCCTGAGGCTTTGGCCAAATGGGCAGAAGGCAGGACAGGTTTTCCTTGGATTGAT 1118
Coturnix_Cry1part       ------------------------------------------------------------
Mus_Cry1                AAGAACCCCGAGGCTCTGGCCAAATGGGCAGAAGGCCGGACAGGCTTCCCGTGGATTGAC 1377
Homo_Cry1               AAAAATCCTGAGGCTTTAGCCAAATGGGCGGAAGGCCGGACAGGCTTTCCATGGATTGAT 1609
                        ** ** ** ****** * ********** ****** ******* ** ** ********

Sylvia_Cry1a            GCAATTATGACACAACTTCGTCAGGAAGGTTGGATTCACCATTTAGCGCGGCATGCTGTA 1083
Gallus_Cry1             GCAATTATGACGCAGCTTCGTCAAGAAGGCTGGATTCACCACTTAGCTCGGCATGCTGTG 1178
Coturnix_Cry1part       ------------------------------------------------------------
Mus_Cry1                GCCATCATGACTCAGCTTCGTCAGGAGGGCTGGATCCACCATTTAGCCAGACACGCGGTT 1437
Homo_Cry1               GCCATCATGACACAGCTTCGTCAGGAGGGTTGGATTCATCATCTAGCCAGGCATGCAGTT 1669
                        ** ** ***** ** ******** ** ** ***** ** **  ****  * ** ** **

Sylvia_Cry1a            GCATGCTTTCTGACTCGAGGTGACCTTTGGATTAGCTGGGAAGAAGGAATGAAGGTCTTT 1143
Gallus_Cry1             GCATGCTTTTTGACTCGAGGTGACCTCTGGATTAGCTGGGAAGAAGGAATGAAGGTCTTT 1238
Coturnix_Cry1part       ------------------------------------------------------------
Mus_Cry1                GCCTGTTTCCTGACTCGTGGTGACCTGTGGATCAGCTGGGAAGAAGGGATGAAGGTCTTT 1497
Homo_Cry1               GCTTGCTTCCTGACACGAGGGGACCTGTGGATTAGTTGGGAAGAAGGAATGAAGGTATTT 1729
                        ** ** **  **** ** ** ***** ***** ** ********** ******** ***
```

```
                                                              p675OL  ►
Sylvia_Cry1a        GAAGAGCTGTTACTTGATGCAGATTGGAGTGTGAATGCTGGAAGCTGGATGTGGCTATCC 1203
Gallus_Cry1         GAAGAGCTCTTACTTGATGCAGATTGGAGTGTGAATGCTGGAAGCTGGATGTGGCTATCC 1298
Coturnix_Cry1part   ------------------------------------------------------------
Mus_Cry1            GAAGAGTTACTGCTTGATGCAGATTGGAGCATAAATGCTGGAAGTTGGATGTGGCTGTCC 1557
Homo_Cry1           GAAGAATTATTGCTTGATGCAGATTGGAGCATAAATGCTGGAAGTTGGATGTGGCTGTCT 1789
                    *****  *  * *****************  * *********** *********** **

Sylvia_Cry1a        TGTAGTTCCTTCTTTCAGCAGTTTTTTCACTGCTACTGCCCAGTGGGTTTTGGCAGAAGA 1263
Gallus_Cry1         TGTAGTTCCTTCTTTCAGCAGTTTTTTCACTGCTACTGTCCAGTGGGTTTTGGCAGAAGA 1358
Coturnix_Cry1part   ------------------------------------------------------------
Mus_Cry1            TGCAGTTCCTTTTTTCAGCAATTTTTTCACTGCTACTGCCCTGTGGGTTTTGGTAGGAGG 1617
Homo_Cry1           TGTAGTTCCTTTTTTCAACAGTTTTTTCACTGCTATTGCCCTGTTGGTTTTGGTAGGAGA 1849
                    ** ******** ***** ** ************** ** ** ** ******** ** **

Sylvia_Cry1a        ACTGACCCAAATGGGGATTATATCAGACGTTATTTACCAGTACTTAGAGGTTTCCCTGCA 1323
Gallus_Cry1         ACTGACCCAAATGGAGATTACATCAGACGTTACTTGCCTGTACTTAGAGGTTTCCCTGCA 1418
Coturnix_Cry1part   ------------------------------------------------------------
Mus_Cry1            ACAGATCCCAATGGAGACTATATTAGGCGTTATTTACCTGTCCTAAGAGGCTTCCCTGCA 1677
Homo_Cry1           ACAGATCCCAATGGAGACTATATCAGGCGTTATTTGCCTGTCCTAAGAGGCTTCCCTGCA 1909
                    ** ** ** ***** ** ** ** ** ***** ** ** ** ** ***** *********

Sylvia_Cry1a        AAATACATCTATGATCCTTGGAATGCCCCAGAGAGCATCCAGAAGGCTGCAAAATGTATC 1383
Gallus_Cry1         AAATACATCTATGATCCTTGGAATGCCCCAGAGAGTGTCCAGAAGGCTGCAAAATGTGTT 1478
Coturnix_Cry1part   ------------------------------------------------------------
Mus_Cry1            AAATATATCTACGATCCTTGGAATGCACCAGAAGGCATCCAGAAGGTTGCCAAGTGTTTG 1737
Homo_Cry1           AAATATATCTATGATCCCTGGAATGCACCAGAAGGTATCCAAAAGGTAGCCAAATGTTTG 1969
                    ***** ***** ***** ******** *****  *  **** ****  ** ** *** *
                                                                  ◄  p678 ....
Sylvia_Cry1a        ATAGGAGTTAATTATCCCAAACCAATGGTAAACCATGCAGAGGCAAGCCGTCTGAATATT 1443
Gallus_Cry1         ATAGGAGTTAACTATCCCAAACCAATGGTAAACCATGCGGAAGCAAGCCGTCTGAATATT 1538
Coturnix_Cry1part   ------------------------------------------------------------
Mus_Cry1            ATAGGAGTTAATTACCCCAAACCGATGGTGAACCATGCTGAGGCAAGCAGACTGAATATT 1797
Homo_Cry1           ATAGGAGTTAATTATCCTAAACCAATGGTGAACCATGCTGAGGCAAGCCGTTTGAATATC 2029
                    *********** ** ** ***** ***** ******** ** ****** *  *******
                 ...                p681        ►
Sylvia_Cry1a        GAGAGGATGAAACAGATCTACCAGCAGCTTTCACGATACAGAGGACTGGGTCTTCTTGCA 1503
Gallus_Cry1         GAGAGGATGAAACAAATCTACCAGCAGCTTTCACGATACAGAGGACTGGGTCTTCTTGCA 1598
Coturnix_Cry1part   ------------------------------------------------------------
Mus_Cry1            GAAAGAATGAAGCAGATCTATCAGCAGCTTTCCCGGTACAGAGGGCTAGGTCTTCTCGCC 1857
Homo_Cry1           GAAAGGATGAAACAGATCTATCAGCAGCTTTCACGATATAGAGGACTAGGTCTTCTGGCA 2089
                    ** ** ***** ** ***** *********** ** ** ***** ** ******** **

Sylvia_Cry1a        ACTGTGCCTTCTAATCCAAATGGAAATGGAAATGGTGGTCTAATGGGCTATTCACCAGGA 1563
Gallus_Cry1         ACAGTGCCTTCTAATCCAAATGGAAATGGAAATGGTGGCCTAATGAGCTTTTCACCAGGA 1658
Coturnix_Cry1part   ------------------------------------------------------------
Mus_Cry1            TCGGTCCCTTCTAACTCTAATGGGAATGGA------GGGCTCATGGGCTATGCTCCTGGA 1911
Homo_Cry1           TCAGTACCTTCTAATCCTAATGGGAATGGA------GGCTTCATGGGATA---TTCTGCA 2140
                     * ** ********  * ***** ******      **  * *** * * *    * * *

Sylvia_Cry1a        GAAAGCATTTCTGGTTGTGGTAGCACAGGAGGAGCTCAGCTGGGAGCTGGCGATGGTCAT 1623
Gallus_Cry1         GAAAGCATTTCTGGTTGCAGCAGTGCAGGAGGAGCTCAGCTGGGAACTGGTGATGGTCAG 1718
Coturnix_Cry1part   ------------------------------------------------------------
Mus_Cry1            GAGAATGTCCCGAGTTGTAGCAGCAGCGGGAATG-------GAGGGCTCATGG------- 1957
Homo_Cry1           GAAAATATCCCAGGTTGTAGCAGCAG---------------------------------- 2166
                    ** *   *  *  ****  * **             ***

Sylvia_Cry1a        TCTGTTG---TTCAGTCATGTGCCCTGGGAGACTCTCATACAGGAACAAGTGGAGTTCAG 1680
Gallus_Cry1         ACAGTTGGCGTTCAGACATGTGCCCTGGGAGACTCTCATACAGGAGGAAGTGGAGTTCAG 1778
Coturnix_Cry1part   ------------------------------------------------------------
Mus_Cry1            --------------GCTATGCTCCTGGAGAGAACGTC--CCGAGCTGTAGCGGTG----- 1996
Homo_Cry1           -----------------------------------------------------TG----- 2168
                                                                      ****

Sylvia_Cry1a        CAGCAAGGTTACTGTCAAGCAAGTAGTATCTTACACTATGCTCATGGAGACAATCAGCAA 1740
Gallus_Cry1         CAGCAAGGTTACTGTCAAGCAAGTAGTATCTTACGCTACGCTCATGGAGACAATCAGCAA 1838
Coturnix_Cry1part   ------------------------------------------------------------
Mus_Cry1            ----GAAATTGCTCTCAAGGAAGTGGTATTTTACACTATGCTCACGGGGACAGTCAGCAG 2052
Homo_Cry1           ----GAAGTTGCTCTCAAGGGAGTGGTATTTTACACTATGCTCATGGCGACAGTCAGCAA 2224
                            ** ** *****  *** **** **** *** ***** ** **** ******
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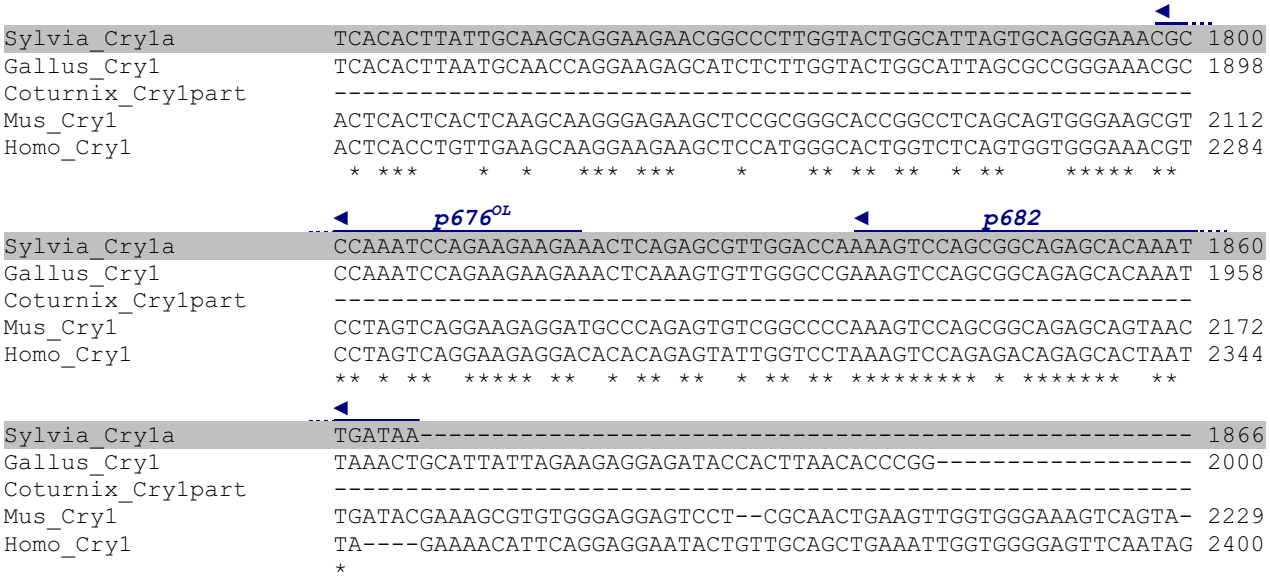


Supplementary **Figure 2: CLUSTAL W (1.83) multiple sequence alignment and details of primer positions used for identification and full length cloning of garden warbler cryptochrome 1a.** The alignment of cryptochrome 1 sequences of *Gallus gallus* [gene bank accession no. AY034432], *Coturnix coturnix* [AF363632] (partial coding sequence), *Mus musculus* [NM007771] & *Homo sapiens* [NM0049705] shows the position of primers used to identify and derive the full length sequence of garden warbler (*Sylvia borin*) cryptochrome 1a. For the sake of completeness, the derived *Sylvia* Cry1a sequence [AJ632120] is included in the alignment.

primer #	primer name	oligonucleotide sequence
p673^{OL}	USP$^{\beta\text{-actin2}}$	5'-GGC ATG TGC AAG GCC GGC TTC-3'
p674^{OL}	DSP$^{\beta\text{-actin4}}$	5'-GGA TGG CAT GAG GGA GCG CGT-3'
p675^{OL}	USP^{Cry1deg}	5'-TTG TCG ACA ATG CTG GAA G(CT)T GGA-3'
p676^{OL}	DSP^{Cry1deg}	5'-TTG AAT TCT TCT TC(CT) (GT)GA (CT)T(AT) GG(AG) CG-3'
p677	SbCry1aNPCRBACK	5'-AGC ACG GTC TCC CAT GGG GGT GAA CGC CGT GCA CTG GTT-3'
p678	SbCry1aNPCRFOR	5'-AGC AAG GTC TCC TTT CAT CCT CTC AAT ATT CAG A-3'
p679	Cry2PCRBACK	5'-AGC ACG TCG ACG AAC CCC ATC TGC ATC CA-3'
p680	Cry2PCRFOR	5'-ACG AAG AAT TCG ACG AAA GCC ACA TCC AG-3'
p681	SbCry1aCPCRBACK	5'-AGC ACG GTC TCC GAA ACA GAT CTA CCA GCA GCT T-3'
p682	SbCry1aCPCRFOR	5'-AGC AAG CGG CCG CTT ATC AAT TTG TGC TCT GCC GCT GGA CTT T-3'
p693	SbCry2PB2	5'-AAT CCC CTG AGA CAG GAA CCC TGA AGC C-3'
p694	SbCry2PB3	5'-AGG AAC CCT GAA GCC TTG GCA AAG-3'
p697	Cry2UTFOR1	5'-TTA TGA GGC CCC CGA TTT TCT GTT G-3'
p698	Cry2UTFOR2	5'-GTT CCT GCT CCT CTG GTC ACC TCT T-3'
p708	SbCry1bCPCRFOR	5'-AGC AAG CGG CCG CTT ATC ATT CTG ATG TTT TGT CTG GTT T-3'
p741	SbCry1.B02	5'-TAG TTC CTT CTT TCA GCA GTT T-3'
p742	SbCry1TruncPCRFOR	5'-AGC AAG CGG CCG CTT ATC AAG TTG CAA GAA GAC CCA GTC CT-3'
p743	SbCry1TruncHisPCRFOR	5'AGC AAG CGG CCG CTT ATC AAT GGT GAT GGT GAT GGT GAG TTG CAA GAA GAC CCA GTC CT-3'

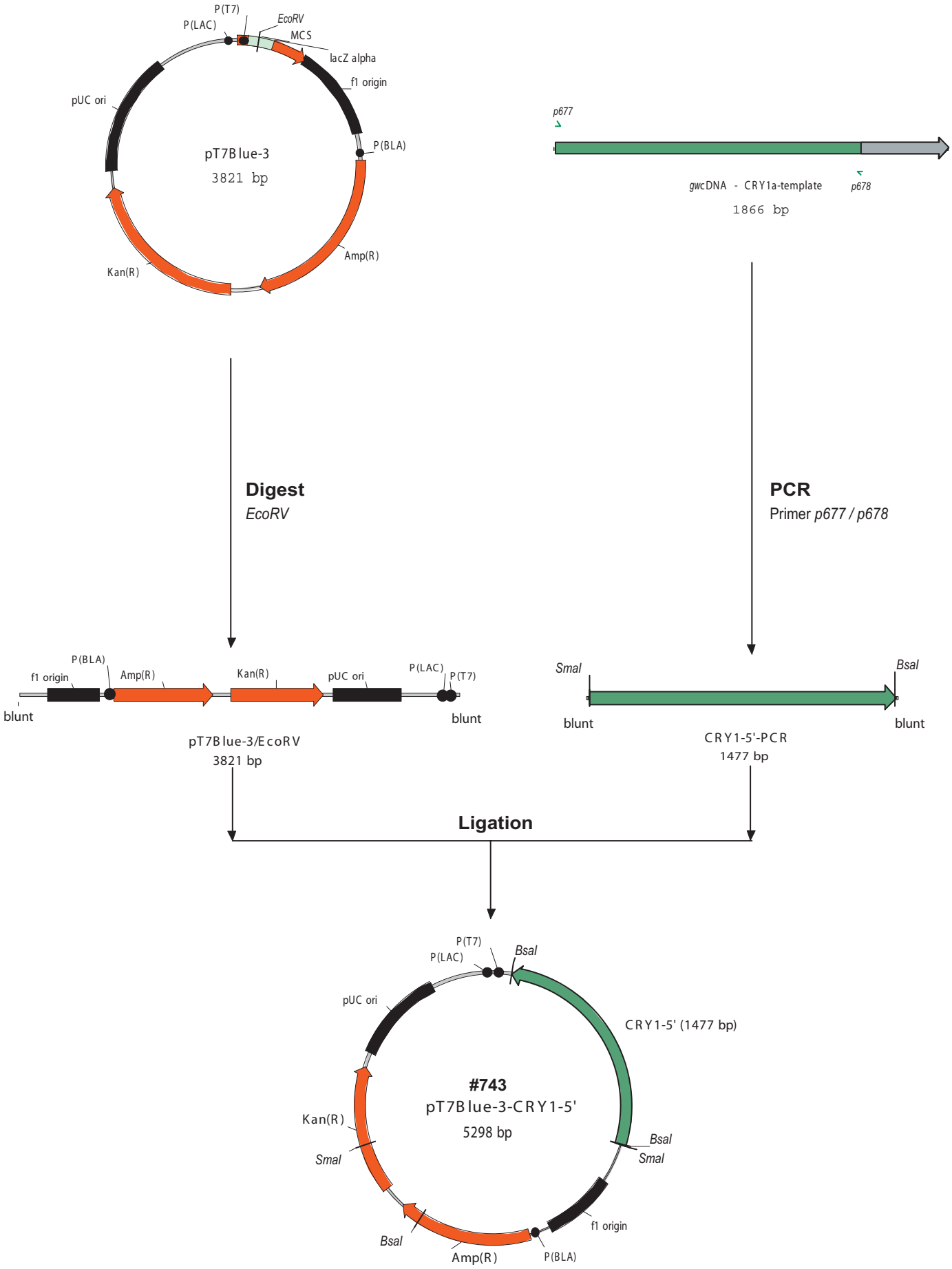
Supplementary **Table 1: Primers used in the garden warbler cryptochrome expression study** A short note on primer naming conventions: *Sb* denotes *Sylvia borin* sequence specific information was available as template for the according primer design. If the primer name does not include *Sb*, the oligonucleotide sequence is derived from an alignment of cryptochrome sequences from other species available in the GenBank data base.

plasmid #	plasmid name	stock bacteria	comment
***gw*Cry1a**			
731	pBC-SK(+)-**CRY1**_intern.fragOL	XL1-Blue MRF'	Garden warbler specific sequence information for cryptochrome 1a
732	pBC-SK(+)-**CRY1**_intern.fragOL	XL1-Blue MRF'	identical to #731
735	pT7Blue-3-**CRY1aN**_T	Nova Blue	consensus sequence for CRY1aN
738	pBC-SK(+)-**CRY1aC**_TP	XL1-Blue MRF'	consensus sequence for CRY1aC
742	pT7Blue-3-**CRY1aN**_T	Nova Blue	consensus sequence for CRY1aN
743	pT7Blue-3-**CRY1aN**_TP	Nova Blue	consensus sequence for CRY1aN, identical to #742
744	pBC-SK(+)-**CRY1aC**_T	XL1-Blue MRF'	consensus sequence for CRY1aC
745	pBC-SK(+)-**CRY1aC**_TP	XL1-Blue MRF'	consensus sequence for CRY1aC, similar to #738,745
752	pDIA92B-NtermHis-**CRY1a_TV**	XL1-Blue MRF'	**BV-Transfer vector -** gwCRY1a-insert: GeneBank accession # **AJ632120**
***gw*Cry1b**			
780	pCR4Blunt-TOPO_**CRY1b**_Cterm	TOP10	***gw*Cry1b** specific C-terminal fragment
781	pCR4Blunt-TOPO_**CRY1b**_Cterm	TOP10	***gw*Cry1b** specific C-terminal fragment; identical to #780
800	pDIA92B-NtermHis-**Cry1B**_TV	XL1-Blue MRF'	**BV-Transfer vector**, gwCRY1b-insert: GeneBank accesion # **DQ838738**
801	pDIA92B-NtermHis-**Cry1B**_TV	XL1-Blue MRF'	BV-Transfer vector, identical to #800
***gw*Cry1PHR**			
874	pDIA92B-NtermHis-**CRY1PHR** 9	XL1-Blue MRF'	BV-Transfer vector
875	pDIA92B-NtermHis-**CRY1PHR** 20	XL1-Blue MRF'	BV-Transfer vector
876	pDIA92B-NtermHis-**CRY1PHR** 22	XL1-Blue MRF'	**BV-Transfer vector**
877	pDIA92B-NtermHis-**CRY1PHR** 24	XL1-Blue MRF'	BV-Transfer vector
878	pDIA92B-**CRY1PHR**-CtermHis 11	XL1-Blue MRF'	BV-Transfer vector
879	pDIA92B-**CRY1PHR**-CtermHis 26	XL1-Blue MRF'	**BV-Transfer vector**
880	pET24d-**CRY1PHR**-CtermHis 3	XL1-Blue MRF'	***E. coli* - Expression vector**
881	pET24d-**CRY1PHR**-CtermHis 5	XL1-Blue MRF'	***E. coli* - Expression vector**
882	pET24d-NtermHis3'RI-**CRY1PHR** 9	XL1-Blue MRF'	***E. coli* - Expression vector**
883	pET24d-NtermHis3'RI-**CRY1PHR** 10	XL1-Blue MRF'	***E. coli* - Expression vector**
***gw*Cry2**			
748	pT7Blue-3-**CRY2**_1	Nova Blue	**Cry2** fragment - from selective colony screen digest.
762	pCR4Blunt-TOPO_**CRY2**_3'UT	TOP10	***gw*CRY2**_3#UT_fragment: GeneBank accession # **AY739908**
763	pCR4Blunt-TOPO_**CRY2**_3'UT	TOP10	identical to #762
***gw*Cry4**			
739	pT7Blue-3-CRY2_1 **CRY4**!	Nova Blue	**CRY4** Derived by cross priming of *p680 & p679* (orientation similar to #740,741)
740	pT7Blue-3-CRY2_2 **CRY4**!	Nova Blue	**CRY4** (orientation similar to #739,741)
741	pT7Blue-3-CRY2_2 **CRY4**!	Nova Blue	**CRY4** (orientation similar to #739,740)
747	pT7Blue-3-CRY2_1 **CRY4**!	Nova Blue	**CRY4** (orientation is reverse to #739,740,741)

Supplementary **Table 2: Summary and breakdown of derived plasmids (numbers and names), relevant for the garden warbler cryptochrome expression study** For the sake of clarity, plasmids, relevant for identification and cloning of *gw*Cry1a, *gw*Cry1b, *gw*Cry1PHR, *gw*Cry2 and *gw*Cry4 are summarised separately. Plasmid numbers of **transfer vectors** (respectively **expression vectors** for *E. coli* expression studies) are highlighted in bold. Plasmid numers on a greyish background denote plasmids, for which a detailed cloning strategy is sketched in this section. 'Stock bacteria' denotes the bacterial strains; plasmids were transformed into and subsequently purified [BV = baculovirus].

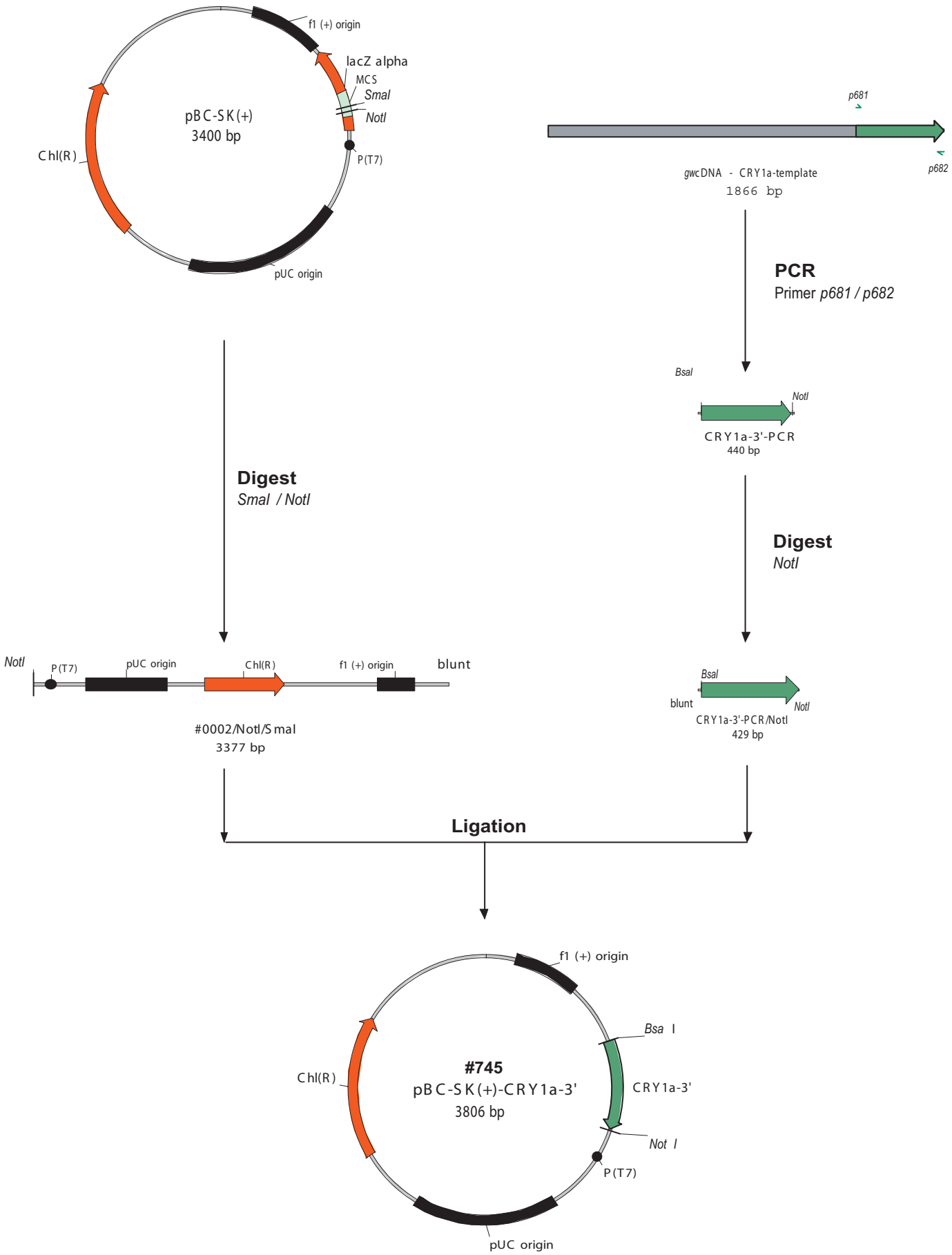
***gw*CRY1a** – cloning strategy, sequence data & protein information

[GenBank accession no. AJ632120]

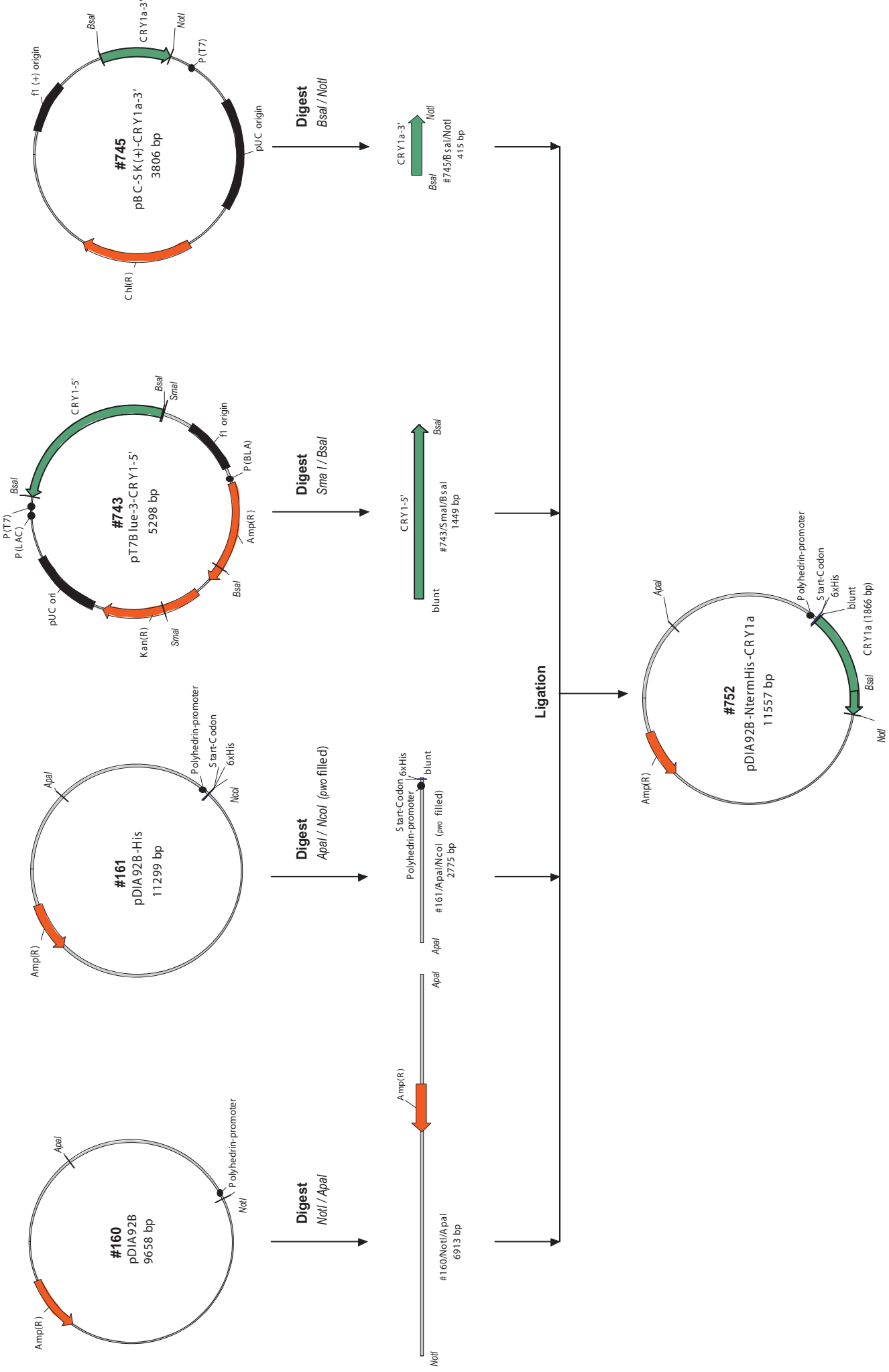


Supplementary **Figure 3: Cloning strategy of the 5' region of garden warbler cryptochrome 1a, *gw*Cry1a (plasmid #743)** The PCR-amplified (primer pair *p677* & *p678*) and blunt-ended fragment was subcloned into an *EcoRV* digested pT7Blue-3 vector. The derived 5' *gw*Cry1 sequence (identical for Cry1a and Cry1b, therefore termed Cry1) is highlighted in green. Theoretical reconstruction of the cloning strategy was performed by means of *VectorNTI*™ (Invitrogen).

Abbreviations: Amp (R)=ampicillin resistance; Chl=chloramphenicol; f1 origin=f1 phage origin of DNA replication; Kan=kanamycin; lacZ alpha=beta galactosidase gene alpha fragment; MCS=multiple cloning site; pUC ori=pUC-derived DNA replication origin; P=promoter; P(bla)=promoter for *bla* gene; P(LAC)=promoter (lac-operon); 6xHis=$(His)_6$-tag.



Supplementary **Figure 4: Cloning strategy of the 3' region of garden warbler cryptochrome 1a, *gw*Cry1a (plasmid #745)** The PCR-amplified fragment (primer pair *p681 & p682*) was digested with *NotI* and ligated as a blunt/*NotI* fragment into *SmaI/NotI* digested pBC-SK(+) vector. The derived 3' *gw*Cry1 sequence is highlighted in green. Theoretical reconstruction of the cloning strategy was performed by means of *VectorNTI™* (Invitrogen). For abbreviations see *Supplementary* Figure 3.



Supplementary **Figure 5: Cloning strategy for transfer vector assembly of full length garden warbler cryptochrome 1a, *gw*Cry1a (plasmid #752)**. Subcloned 5' and 3' fragments of the garden warbler Cry1a (#743 & #745) were assembled in a pVL1392-derived baculovirus transfer vector, which allowed for subsequent recombinant expression of garden warbler Cry1a in a *Sf9*/baculovirus expression system. The derived *gw*Cry1a sequence is highlighted in green. Theoretical reconstruction of the cloning strategy was performed by means of *VectorNTI™* (Invitrogen). For abbreviations see *Supplementary* Figure 3.

Sequence data

*gw*CRY1a

Plasmid number	**#752**
Plasmid name	**pDIA92B-NtermHis-CRY1a**
Nucleic acid sequence	**1884 bp** (including $(His)_6$-tag)
Amino acid sequence	**628 aa**

```
            ____(His)6-tag____
              M  H  H  H  H  H  H   A  M  G   V  N  A   V  H  W  F ·
4151    ATGCACCAT CACCATCACC ATGCCATGGG GGTGAACGCC GTGCACTGGT

        · R  K  G   L  R  L   H  D  N  P   A  L  R   E  C  I
4201    TCCGCAAGGG GCTGCGGCTC CACGACAACC CCGCGCTGCG GGAATGCATC

         Q  G  A  D   T  V  R   C  V  Y   I  L  D  P   W  F  A ·
4251    CAGGGCGCCG ACACGGTGCG CTGCGTCTAC ATCCTGGACC CCTGGTTCGC

        · G  S  S   N  V  G  I   N  R  W   R  F  L   L  Q  C  L ·
4301    CGGCTCCTCC AACGTGGGCA TCAACAGGTG GCGATTCCTG CTTCAATGTC

        · E  D  L   D  A  N   L  R  K  L   N  S  R   L  F  V
4351    TTGAGGATCT CGATGCCAAT CTGCGGAAAC TGAATTCACG TTTGTTTGTT

         I  R  G  Q   P  A  D   V  F  P   R  L  F  K   E  W  N ·
4401    ATTCGTGGAC AGCCAGCAGA TGTTTTCCCC AGGCTTTTTA AGGAATGGAA

        · I  A  K   L  S  I  E   Y  D  S   E  P  F   G  K  E  R ·
4451    CATTGCAAAA CTTTCTATTG AATATGATTC TGAACCATTT GGGAAGGAAA

        · D  A  A   I  K  K   L  A  S  E   A  G  V   E  V  I
4501    GAGATGCAGC TATCAAGAAG CTGGCTAGTG AAGCTGGAGT GGAGGTCATT

         V  R  I  S   H  T  L   Y  D  L   D  K  I  I   E  L  N ·
4551    GTTCGGATTT CTCATACGTT GTATGACCTA GACAAAATAA TAGAATTAAA

        · G  G  Q   P  P  L  T   Y  K  R   F  Q  T   L  I  S  R ·
4601    TGGAGGACAG CCTCCTCTTA CTTACAAGCG ATTTCAGACC CTAATTAGTA

        · M  E  P   L  E  M   P  V  E  T   I  T  P   E  V  M
4651    GAATGGAACC CCTGGAGATG CCTGTGGAGA CTATTACCCC AGAAGTAATG

         K  K  C  T   T  P  V   S  D  D   H  D  E  K   Y  G  V ·
4701    AAAAAATGCA CTACTCCAGT TTCTGATGAT CACGATGAGA AATACGGTGT

        · P  S  L   E  E  L  G   F  D  T   D  G  L   P  S  A  V ·
4751    GCCATCCCTT GAAGAGCTGG GTTTTGACAC GGATGGTCTG CCTTCTGCAG

        · W  P  G   G  E  T   E  A  L  T   R  L  E   R  H  L
4801    TATGGCCAGG GGGAGAAACT GAAGCTCTCA CACGATTAGA AAGACATTTA
```

```
         E  R  K  A  W  V  A  N  F  E  R  P  R  M  N  A  N·
4851  GAACGAAAGG CTTGGGTAGC AAACTTTGAA AGACCACGAA TGAATGCCAA

      ·S  L  L  A  S  P  T  G  L  S  P  Y  L  R  F  G  C·
4901  TTCCCTTCTG GCAAGCCCTA CGGGGCTCAG CCCCTACCTC CGCTTTGGCT

      ·L  S  C  R  L  F  Y  F  K  L  T  D  L  Y  K  K
4951  GTTTGTCCTG TCGGCTCTTT TATTTCAAGT TAACGGATCT GTACAAAAAG

       V  K  K  N  S  S  P  P  L  S  L  Y  G  Q  L  L  W·
5001  GTAAAAAAGA ACAGCTCCCC TCCCCTCTCC CTCTATGGCC AGCTGTTATG

      ·R  E  F  F  Y  T  A  A  T  N  N  P  R  F  D  K  M·
5051  GCGTGAATTT TTCTACACAG CGGCGACTAA CAATCCACGG TTTGATAAAA

      ·E  G  N  P  I  C  V  Q  I  P  W  D  K  N  P  E
5101  TGGAGGGGAA TCCTATCTGT GTTCAAATTC CGTGGGATAA GAATCCTGAG

       A  L  A  K  W  A  E  G  R  T  G  F  P  W  I  D  A·
5151  GCTTTGGCCA AATGGGCAGA AGGCAGGACA GGTTTTCCTT GGATTGATGC

      ·I  M  T  Q  L  R  Q  E  G  W  I  H  H  L  A  R  H·
5201  AATTATGACA CAACTTCGTC AGGAAGGTTG GATTCACCAT TTAGCGCGGC

      ·A  V  A  C  F  L  T  R  G  D  L  W  I  S  W  E
5251  ATGCTGTAGC ATGCTTTCTG ACTCGAGGTG ACCTTTGGAT TAGCTGGGAA

       E  G  M  K  V  F  E  E  L  L  L  D  A  D  W  S  V·
5301  GAAGGAATGA AGGTCTTTGA AGAGCTGTTA CTTGATGCAG ATTGGAGTGT

      ·N  A  G  S  W  M  W  L  S  C  S  S  F  F  Q  Q  F·
5351  GAATGCTGGA AGCTGGATGT GGCTATCCTG TAGTTCCTTC TTTCAGCAGT

      ·F  H  C  Y  C  P  V  G  F  G  R  R  T  D  P  N
5401  TTTTTCACTG CTACTGCCCA GTGGGTTTTG GCAGAAGAAC TGACCCAAAT

       G  D  Y  I  R  R  Y  L  P  V  L  R  G  F  P  A  K·
5451  GGGGATTATA TCAGACGTTA TTTACCAGTA CTTAGAGGTT TCCCTGCAAA

      ·Y  I  Y  D  P  W  N  A  P  E  S  I  Q  K  A  A  K·
5501  ATACATCTAT GATCCTTGGA ATGCCCCAGA GAGCATCCAG AAGGCTGCAA

      ·C  I  I  G  V  N  Y  P  K  P  M  V  N  H  A  E
5551  AATGTATCAT AGGAGTTAAT TATCCCAAAC CAATGGTAAA CCATGCAGAG

       A  S  R  L  N  I  E  R  M  K  Q  I  Y  Q  Q  L  S·
5601  GCAAGCCGTC TGAATATTGA GAGGATGAAA CAGATCTACC AGCAGCTTTC

      ·R  Y  R  G  L  G  L  L  A  T  V  P  S  N  P  N  G·
5651  ACGATACAGA GGACTGGGTC TTCTTGCAAC TGTGCCTTCT AATCCAAATG


      ·N  G  N  G  G  L  M  G  Y  S  P  G  E  S  I  S
5701  GAAATGGAAA TGGTGGTCTA ATGGGCTATT CACCAGGAGA AAGCATTTCT

       G  C  G  S  T  G  G  A  Q  L  G  A  G  D  G  H  S·
5751  GGTTGTGGTA GCACAGGAGG AGCTCAGCTG GGAGCTGGCG ATGGTCATTC
```

```
      · V  V  Q   S  C  A  L   G  D  S   H  T  G   T  S  G  V ·
5801  TGTTGTTCAG TCATGTGCCC TGGGAGACTC TCATACAGGA ACAAGTGGAG

      · Q  Q  Q   G  Y  C  Q   A  S  S   I  L  H   Y  A  H
5851  TTCAGCAGCA AGGTTACTGT CAAGCAAGTA GTATCTTACA CTATGCTCAT

       G  D  N  Q   Q  S  H   L  L  Q   A  G  R  T   A  L  G ·
5901  GGAGACAATC AGCAATCACA CTTATTGCAA GCAGGAAGAA CGGCCCTTGG

      · T  G  I   S  A  G  K   R  P  N   P  E  E   E  T  Q  S ·
5951  TACTGGCATT AGTGCAGGGA AACGCCCAAA TCCAGAAGAA GAAACTCAGA

      · V  G  P   K  V  Q   R  Q  S  T   N  *  *
6001  GCGTTGGACC AAAAGTCCAG CGGCAGAGCA CAAATTGATAA
```

Protein data

*gw*CRY1a

Plasmid number **#752**

Plasmid name **pDIA92B-NtermHis-CRY1a**

Amino acid sequence **628 aa**

analysis	*gw*Cry1a
Length	628 aa
Molecular Weight	70594.57 m.w.
1 µg =	14.165 pMoles
Molar Extinction coefficient	125410
1 A[280] corr. to	0.56 mg/ml
A[280] of 1 mg/ml	1.78 AU
Isoelectric Point	8.11
Charge at pH 7	4.53

amino acid(s)	number count	% by weight	% by frequency
Charged (RKHYCDE)	190	35.51	30.25
Acidic (DE)	65	11.20	10.35
Basic (KR)	68	13.47	10.83
Polar (NCQSTY)	161	25.68	25.64
Hydrophobic (AILFWV)	207	32.92	32.96

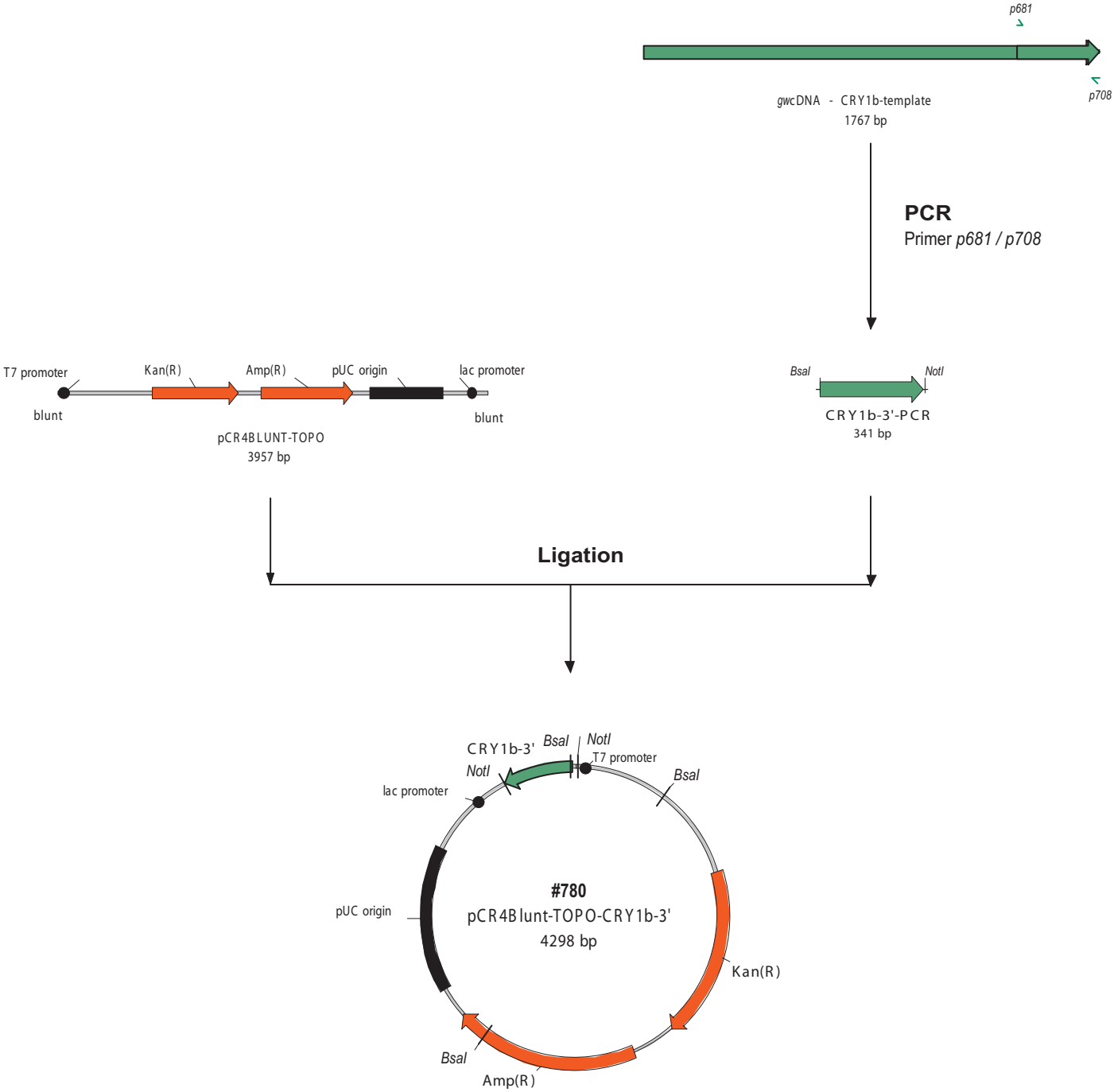
amino acid	number count	% by weight	% by frequency
A Ala	44	4.79	7.01
C Cys	15	2.22	2.39
D Asp	28	4.55	4.46
E Glu	37	6.65	5.89
F Phe	25	5.04	3.98
G Gly	55	5.04	8.76
H His	21	3.98	3.34
I Ile	29	4.65	4.62
K Lys	29	5.18	4.62
L Leu	61	9.77	9.71
M Met	13	2.37	2.07
N Asn	29	4.68	4.62
P Pro	38	5.34	6.05
Q Gln	27	4.82	4.30
R Arg	39	8.30	6.21
S Ser	42	5.39	6.69
T Thr	27	3.93	4.30
V Val	31	4.43	4.94
W Trp	17	4.24	2.71
Y Tyr	21	4.64	3.34
B Asx	57	9.23	9.08
Z Glx	64	11.47	10.19
X Xxx	0	0.00	0.00

Aminoacid sequence:

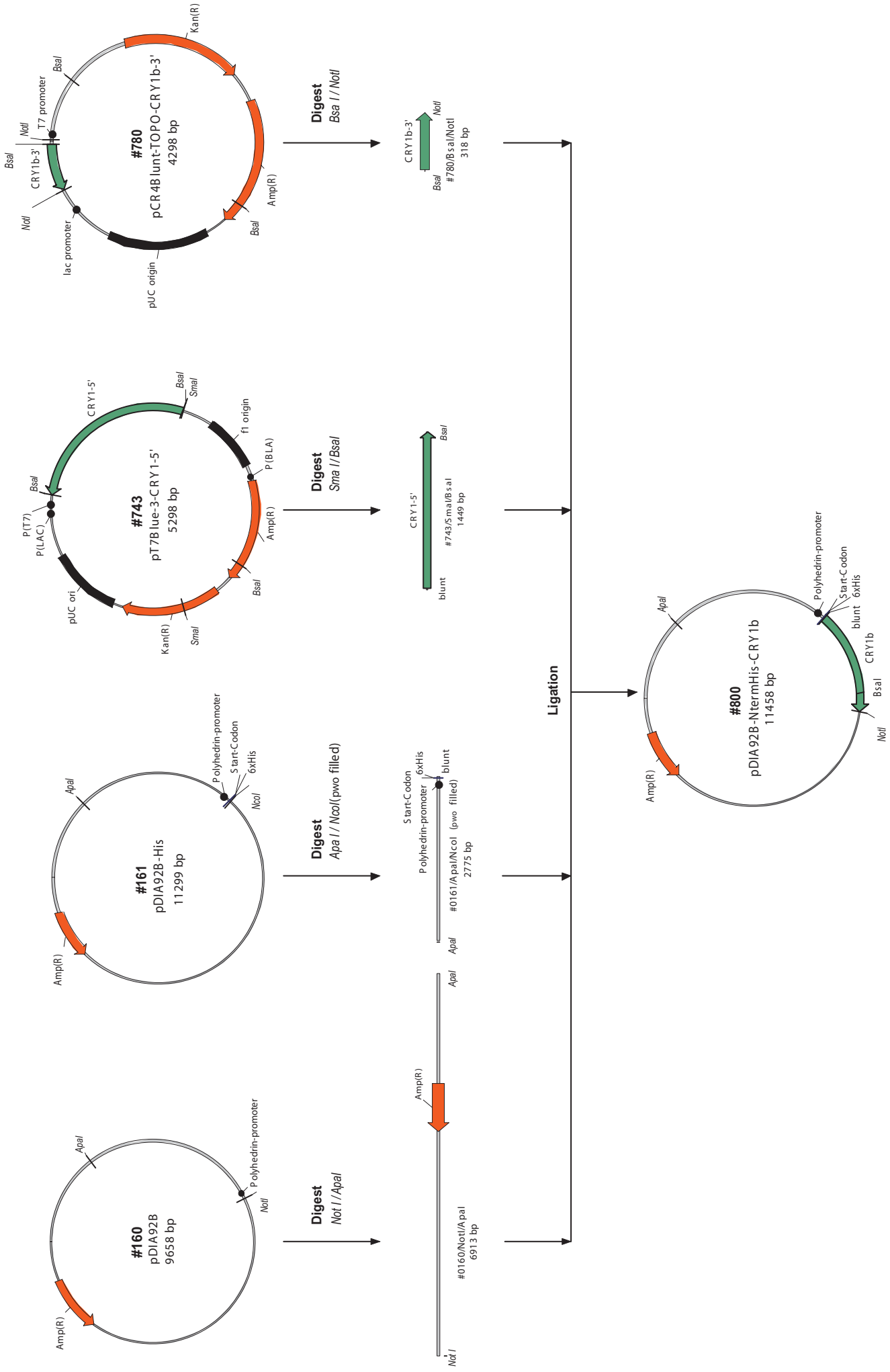
MHHHHHHAMGVNAVHWFRKGLRLHDNPALRECIQ
GADTVRCVYILDPWFAGSSNVGINRWRFLLQCLE
DLDANLRKLNSRLFVIRGQPADVFPRLFKEWNIA
KLSIEYDSEPFGKERDAAIKKLASEAGVEVIVRI
SHTLYDLDKIIELNGGQPPLTYKRFQTLISRMEP
LEMPVETITPEVMKKCTTPVSDDHDEKYGVPSLE
ELGFDTDGLPSAVWPGGETEALTRLERHLERKAW
VANFERPRMNANSLLASPTGLSPYLRFGCLSCRL
FYFKLTDLYKKVKKNSSPPLSLYGQLLWREFFYT
AATNNPRFDKMEGNPICVQIPWDKNPEALAKWAE
GRTGFPWIDAIMTQLRQEGWIHHLARHAVACFLT
RGDLWISWEEGMKVFEELLLDADWSVNAGSWMWL
SCSSFFQQFFHCYCPVGFGRRTDPNGDYIRRYLP
VLRGFPAKYIYDPWNAPESIQKAAKCIIGVNYPK
PMVNHAEASRLNIERMKQIYQQLSRYRGLGLLAT
VPSNPNGNGNGGLMGYSPGESISGCGSTGGAQLG
AGDGHSVVQSCALGDSHTGTSGVQQQGYCQASSI
LHYAHGDNQQSHLLQAGRTALGTGISAGKRPNPE
EETQSVGPKVQRQSTN

***gw*CRY1b** – cloning strategy, sequence data & protein information

[GenBank accession no. DQ838738]



Supplementary **Figure 6: Cloning strategy of the garden warbler cryptochrome 1b specific 3' region (plasmid #780)** The PCR-amplified fragment (primer pair *p681* & *p708*) was cloned into the blunt vector pCR4BLUNT-TOPO, yielding the intermediate plasmid #780. The derived garden warbler specific 3' *gw*Cry1b sequence is highlighted in green. Theoretical reconstruction of the cloning strategy was performed by means of *VectorNTI™* (Invitrogen). For abbreviations see *Supplementary* Figure 3.



Supplementary **Figure 7: Cloning strategy for transfer vector assembly of full length garden warbler cryptochrome 1b, *gw*Cry1b (plasmid #800)** Subcloned 5' and 3' fragments of the garden warbler Cry1b (#743 & #780) were assembled in a pVL1392-derived baculovirus transfer vector, which allowed for subsequent recombinant expression of garden warbler Cry1a in a *Sf9*/baculovirus expression system. The derived *gw*Cry1a sequence is highlighted in green. Theoretical reconstruction of the cloning strategy was performed by means of *VectorNTI™* (Invitrogen). For abbreviations see *Supplementary* Figure 3.

Sequence data

*gw*CRY1b

Plasmid number	**#800**
Plasmid name	**pDIA92B-NtermHis-CRY1b**
Nucleic acid sequence	**1785 bp** (including $(His)_6$-tag)
Amino acid sequence	**595 aa**

```
          ____(His)6-tag____
            M  H  H  H  H  H  H   A  M  G   V  N  A   V  H  W  F ·
4151    ATGCACCAT CACCATCACC ATGCCATGGG GGTGAACGCC GTGCACTGGT

        · R  K  G   L  R  L   H  D  N  P   A  L  R   E  C  I
4201    TCCGCAAGGG GCTGCGGCTC CACGACAACC CCGCGCTGCG GGAATGCATC

         Q  G  A  D   T  V  R   C  V  Y   I  L  D  P   W  F  A ·
4251    CAGGGCGCCG ACACGGTGCG CTGCGTCTAC ATCCTGGACC CCTGGTTCGC

        · G  S  S   N  V  G  I   N  R  W   R  F  L   L  Q  C  L ·
4301    CGGCTCCTCC AACGTGGGCA TCAACAGGTG GCGATTCCTG CTTCAATGTC

        · E  D  L   D  A  N   L  R  K  L   N  S  R   L  F  V
4351    TTGAGGATCT CGATGCCAAT CTGCGGAAAC TGAATTCACG TTTGTTTGTT

         I  R  G  Q   P  A  D   V  F  P   R  L  F  K   E  W  N ·
4401    ATTCGTGGAC AGCCAGCAGA TGTTTTCCCC AGGCTTTTTA AGGAATGGAA

        · I  A  K   L  S  I  E   Y  D  S   E  P  F   G  K  E  R ·
4451    CATTGCAAAA CTTTCTATTG AATATGATTC TGAACCATTT GGGAAGGAAA

        · D  A  A   I  K  K   L  A  S  E   A  G  V   E  V  I
4501    GAGATGCAGC TATCAAGAAG CTGGCTAGTG AAGCTGGAGT GGAGGTCATT

         V  R  I  S   H  T  L   Y  D  L   D  K  I  I   E  L  N ·
4551    GTTCGGATTT CTCATACGTT GTATGACCTA GACAAAATAA TAGAATTAAA

        · G  G  Q   P  P  L  T   Y  K  R   F  Q  T   L  I  S  R ·
4601    TGGAGGACAG CCTCCTCTTA CTTACAAGCG ATTTCAGACC CTAATTAGTA

        · M  E  P   L  E  M   P  V  E  T   I  T  P   E  V  M
4651    GAATGGAACC CCTGGAGATG CCTGTGGAGA CTATTACCCC AGAAGTAATG

         K  K  C  T   T  P  V   S  D  D   H  D  E  K   Y  G  V ·
4701    AAAAAATGCA CTACTCCAGT TTCTGATGAT CACGATGAGA AATACGGTGT

        · P  S  L   E  E  L  G   F  D  T   D  G  L   P  S  A  V ·
4751    GCCATCCCTT GAAGAGCTGG GTTTTGACAC GGATGGTCTG CCTTCTGCAG

        · W  P  G   G  E  T   E  A  L  T   R  L  E   R  H  L
4801    TATGGCCAGG GGGAGAAACT GAAGCTCTCA CACGATTAGA AAGACATTTA
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         E  R  K  A  W  V  A  N  F  E  R  P  R  M  N  A  N·
4851  GAACGAAAGG CTTGGGTAGC AAACTTTGAA AGACCACGAA TGAATGCCAA

      ·S  L  L  A  S  P  T  G  L  S  P  Y  L  R  F  G  C·
4901  TTCCCTTCTG GCAAGCCCTA CGGGGCTCAG CCCCTACCTC CGCTTTGGCT

      ·  L  S  C  R  L  F  Y  F  K  L  T  D  L  Y  K  K
4951  GTTTGTCCTG TCGGCTCTTT TATTTCAAGT TAACGGATCT GTACAAAAAG

       V  K  K  N  S  S  P  P  L  S  L  Y  G  Q  L  L  W·
5001  GTAAAAAAGA ACAGCTCCCC TCCCCTCTCC CTCTATGGCC AGCTGTTATG

      ·R  E  F  F  Y  T  A  A  T  N  N  P  R  F  D  K  M·
5051  GCGTGAATTT TTCTACACAG CGGCGACTAA CAATCCACGG TTTGATAAAA

      ·  E  G  N  P  I  C  V  Q  I  P  W  D  K  N  P  E
5101  TGGAGGGGAA TCCTATCTGT GTTCAAATTC CGTGGGATAA GAATCCTGAG

       A  L  A  K  W  A  E  G  R  T  G  F  P  W  I  D  A·
5151  GCTTTGGCCA AATGGGCAGA AGGCAGGACA GGTTTTCCTT GGATTGATGC

      ·I  M  T  Q  L  R  Q  E  G  W  I  H  H  L  A  R  H·
5201  AATTATGACA CAACTTCGTC AGGAAGGTTG GATTCACCAT TTAGCGCGGC

      ·  A  V  A  C  F  L  T  R  G  D  L  W  I  S  W  E
5251  ATGCTGTAGC ATGCTTTCTG ACTCGAGGTG ACCTTTGGAT TAGCTGGGAA

       E  G  M  K  V  F  E  E  L  L  L  D  A  D  W  S  V·
5301  GAAGGAATGA AGGTCTTTGA AGAGCTGTTA CTTGATGCAG ATTGGAGTGT

      ·N  A  G  S  W  M  W  L  S  C  S  S  F  F  Q  Q  F·
5351  GAATGCTGGA AGCTGGATGT GGCTATCCTG TAGTTCCTTC TTTCAGCAGT

      ·  F  H  C  Y  C  P  V  G  F  G  R  R  T  D  P  N
5401  TTTTTCACTG CTACTGCCCA GTGGGTTTTG GCAGAAGAAC TGACCCAAAT

       G  D  Y  I  R  R  Y  L  P  V  L  R  G  F  P  A  K·
5451  GGGGATTATA TCAGACGTTA TTTACCAGTA CTTAGAGGTT TCCCTGCAAA

      ·Y  I  Y  D  P  W  N  A  P  E  S  I  Q  K  A  A  K·
5501  ATACATCTAT GATCCTTGGA ATGCCCCAGA GAGCATCCAG AAGGCTGCAA

      ·  C  I  I  G  V  N  Y  P  K  P  M  V  N  H  A  E
5551  AATGTATCAT AGGAGTTAAT TATCCCAAAC CAATGGTAAA CCATGCAGAG

       A  S  R  L  N  I  E  R  M  K  Q  I  Y  Q  Q  L  S·
5601  GCAAGCCGTC TGAATATTGA GAGGATGAAA CAGATCTACC AGCAGCTTTC

      ·R  Y  R  G  L  G  L  L  A  T  V  P  S  N  P  N  G·
5651  ACGATACAGA GGACTGGGTC TTCTTGCAAC TGTGCCTTCT AATCCAAATG


      ·  N  G  N  G  G  L  M  G  Y  S  P  G  E  S  I  S
5701  GAAATGGAAA TGGTGGTCTA ATGGGCTATT CACCAGGAGA AAGCATTTCT

       G  C  G  S  T  G  G  A  Q  L  G  A  G  D  G  H  S·
5751  GGTTGTGGTA GCACAGGAGG AGCTCAGCTG GGAGCTGGCG ATGGTCATTC
```

```
        · V  V  Q   S  C  A  L   G  D  S   H  T  G   T  S  G  V ·
5801    TGTTGTTCAG TCATGTGCCC TGGGAGACTC TCATACAGGA ACAAGTGGAG

        · Q  Q  Q   G  I  V   A  V  A  V   C  R  G   S  P  N
5851    TTCAGCAGCA AGGTATTGTG GCAGTGGCTG TCTGTAGAGG CTCTCCAAAT

         P  C  N  Y   G  K  P   D  K  T   S  E  *  *
5901    CCTTGCAACT ATGGAAAACC AGACAAAACA TCAGAATGAT AA
```

Protein data

*gw*CRY1b

Plasmid number **#800, #801**

Plasmid name **pDIA92B-NtermHis-CRY1b**

Amino acid sequence **595 aa**

analysis	*gw*Cry1b
Length	595 aa
Molecular Weight	66981.97 m.w.
1 µg =	14.929 pMoles
Molar Extinction coefficient	124250
1 A[280] corr. to	0.54 mg/ml
A[280] of 1 mg/ml	1.85 AU
Isoelectric Point	8.09
Charge at pH 7	4.25

amino acid(s)	number count	% by weight	% by frequency
Charged (RKHYCDE)	183	35.93	30.76
Acidic (DE)	63	11.43	10.59
Basic (KR)	66	13.75	11.09
Polar (NCQSTY)	146	24.50	24.54
Hydrophobic (AILFWV)	200	33.67	33.61

amino acid	number count	% by weight	% by frequency
A Ala	41	4.70	6.89
C Cys	16	2.50	2.69
D Asp	28	4.80	4.71
E Glu	35	6.63	5.88
F Phe	25	5.32	4.20
G Gly	51	4.93	8.57
H His	18	3.60	3.03
I Ile	28	4.73	4.71
K Lys	29	5.46	4.87
L Leu	57	9.62	9.58
M Met	13	2.50	2.18
N Asn	28	4.76	4.71
P Pro	38	5.63	6.39
Q Gln	20	3.76	3.36
R Arg	37	8.30	6.22
S Ser	38	5.14	6.39
T Thr	24	3.68	4.03
V Val	32	4.83	5.38
W Trp	17	4.47	2.86
Y Tyr	20	4.66	3.36
B Asx	56	9.56	9.41
Z Glx	55	10.39	9.24
X Xxx	0	0.00	0.00

Aminoacid sequence:

MHHHHHHAMGVNAVHWFRKGLRLHDNPALRECIQ
GADTVRCVYILDPWFAGSSNVGINRWRFLLQCLE
DLDANLRKLNSRLFVIRGQPADVFPRLFKEWNIA
KLSIEYDSEPFGKERDAAIKKLASEAGVEVIVRI
SHTLYDLDKIIELNGGQPPLTYKRFQTLISRMEP
LEMPVETITPEVMKKCTTPVSDDHDEKYGVPSLE
ELGFDTDGLPSAVWPGGETEALTRLERHLERKAW
VANFERPRMNANSLLASPTGLSPYLRFGCLSCRL
FYFKLTDLYKKVKKNSSPPLSLYGQLLWREFFYT
AATNNPRFDKMEGNPICVQIPWDKNPEALAKWAE
GRTGFPWIDAIMTQLRQEGWIHHLARHAVACFLT
RGDLWISWEEGMKVFEELLLDADWSVNAGSWMWL
SCSSFFQQFFHCYCPVGFGRRTDPNGDYIRRYLP
VLRGFPAKYIYDPWNAPESIQKAAKCIIGVNYPK
PMVNHAEASRLNIERMKQIYQQLSRYRGLGLLAT
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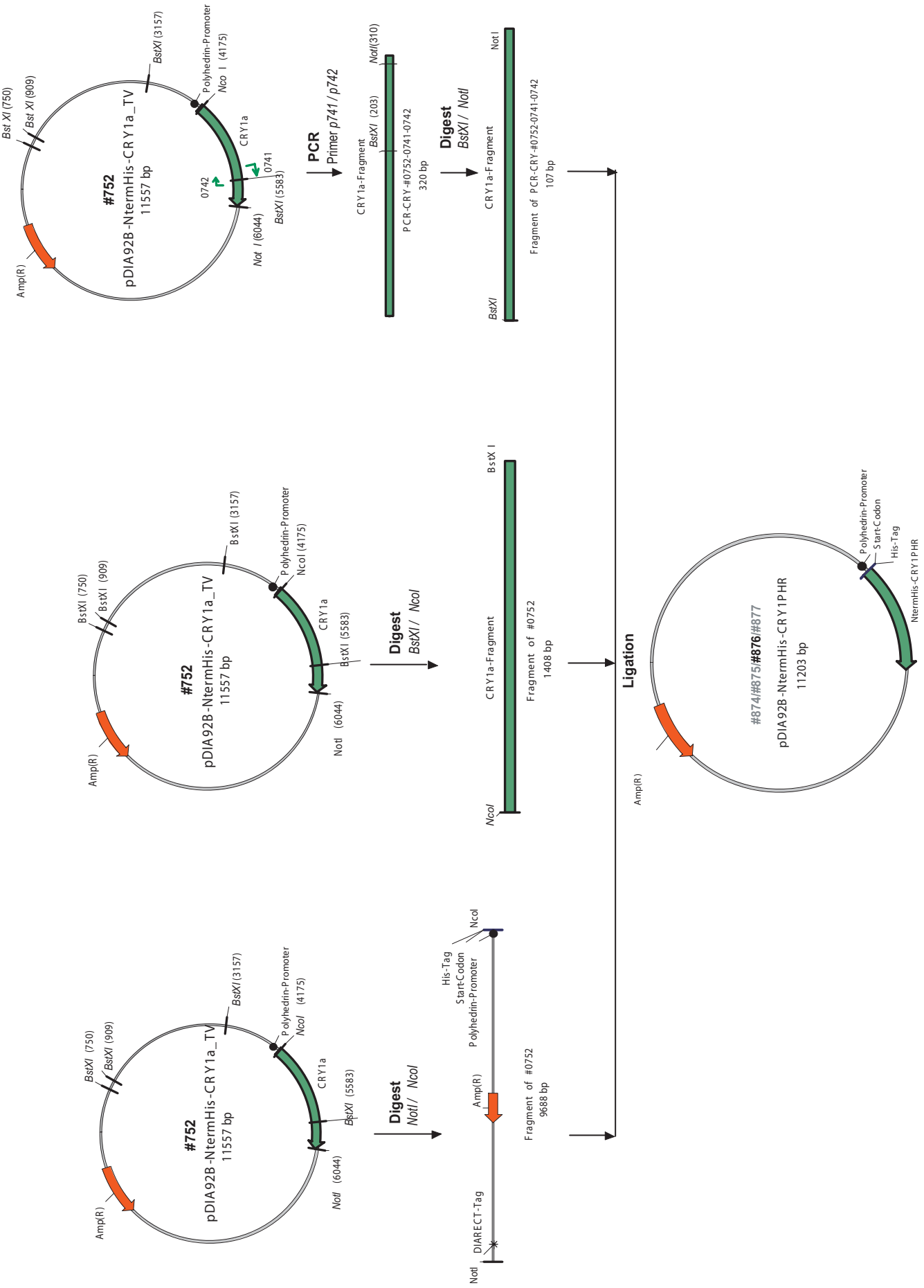

Supplementary **Figure 8: Cloning strategy of the garden warbler photolyase homology region (PHR) (plasmid #876), baculovirus transfervector, N-terminal (His)$_6$-tag** The PCR-amplified fragment (primer pair *p741 & p742*) was digested with *BstXI*/*NotI*, resulting in a 107 bp target fragment. This fragment was used to exchange the Cry1a C-terminus of transfer vector #752 (see *Suppl. I* Figure 5) against the PHR C-terminus. Theoretical reconstruction of the cloning strategy was performed by means of *VectorNT™* (Invitrogen). For abbreviations see *Supplementary* Figure 3.

Sequence data

*gw*CRY1PHR

Plasmid number	#874, #875, **#876**, #877
Plasmid name	**pDIA92B- NtermHis-CRY1PHR**
Nucleic acid sequence	**1536 bp** (including $(His)_6$-tag)
Amino acid sequence	**510 aa**

```
          (His)6-tag
      M  H  H  H  H  H  H   A  M  G   V  N  A   V  H  W  F ·
4151   ATGCACCAT CACCATCACC ATGCCATGGG GGTGAACGCC GTGCACTGGT
   ·  R  K  G  L  R  L  H  D  N  P  A  L  R  E  C  I
4201   TCCGCAAGGG GCTGCGGCTC CACGACAACC CCGCGCTGCG GGAATGCATC
    Q  G  A  D  T  V  R  C  V  Y  I  L  D  P  W  F  A ·
4251   CAGGGCGCCG ACACGGTGCG CTGCGTCTAC ATCCTGGACC CCTGGTTCGC
   · G  S  S  N  V  G  I  N  R  W  R  F  L  L  Q  C  L ·
4301   CGGCTCCTCC AACGTGGGCA TCAACAGGTG GCGATTCCTG CTTCAATGTC
   ·  E  D  L  D  A  N  L  R  K  L  N  S  R  L  F  V
4351   TTGAGGATCT CGATGCCAAT CTGCGGAAAC TGAATTCACG TTTGTTTGTT
    I  R  G  Q  P  A  D  V  F  P  R  L  F  K  E  W  N ·
4401   ATTCGTGGAC AGCCAGCAGA TGTTTTCCCC AGGCTTTTTA AGGAATGGAA
   · I  A  K  L  S  I  E  Y  D  S  E  P  F  G  K  E  R ·
4451   CATTGCAAAA CTTTCTATTG AATATGATTC TGAACCATTT GGGAAGGAAA
   ·  D  A  A  I  K  K  L  A  S  E  A  G  V  E  V  I
4501   GAGATGCAGC TATCAAGAAG CTGGCTAGTG AAGCTGGAGT GGAGGTCATT
    V  R  I  S  H  T  L  Y  D  L  D  K  I  I  E  L  N ·
4551   GTTCGGATTT CTCATACGTT GTATGACCTA GACAAAATAA TAGAATTAAA
   · G  G  Q  P  P  L  T  Y  K  R  F  Q  T  L  I  S  R ·
4601   TGGAGGACAG CCTCCTCTTA CTTACAAGCG ATTTCAGACC CTAATTAGTA
   ·  M  E  P  L  E  M  P  V  E  T  I  T  P  E  V  M
4651   GAATGGAACC CCTGGAGATG CCTGTGGAGA CTATTACCCC AGAAGTAATG
    K  K  C  T  T  P  V  S  D  D  H  D  E  K  Y  G  V ·
4701   AAAAAATGCA CTACTCCAGT TTCTGATGAT CACGATGAGA AATACGGTGT
   · P  S  L  E  E  L  G  F  D  T  D  G  L  P  S  A  V ·
4751   GCCATCCCTT GAAGAGCTGG GTTTTGACAC GGATGGTCTG CCTTCTGCAG
   ·  W  P  G  G  E  T  E  A  L  T  R  L  E  R  H  L
4801   TATGGCCAGG GGGAGAAACT GAAGCTCTCA CACGATTAGA AAGACATTTA
```

```
    E  R  K  A  W  V  A  N  F  E  R  P  R  M  N  A  N·
4851  GAACGAAAGG CTTGGGTAGC AAACTTTGAA AGACCACGAA TGAATGCCAA
   ·S  L  L  A  S  P  T  G  L  S  P  Y  L  R  F  G  C·
4901  TTCCCTTCTG GCAAGCCCTA CGGGGCTCAG CCCCTACCTC CGCTTTGGCT
   ·  L  S  C  R  L  F  Y  F  K  L  T  D  L  Y  K  K
4951  GTTTGTCCTG TCGGCTCTTT TATTTCAAGT TAACGGATCT GTACAAAAAG
    V  K  K  N  S  S  P  P  L  S  L  Y  G  Q  L  L  W·
5001  GTAAAAAAGA ACAGCTCCCC TCCCCTCTCC CTCTATGGCC AGCTGTTATG
   ·R  E  F  F  Y  T  A  A  T  N  N  P  R  F  D  K  M·
5051  GCGTGAATTT TTCTACACAG CGGCGACTAA CAATCCACGG TTTGATAAAA
   ·  E  G  N  P  I  C  V  Q  I  P  W  D  K  N  P  E
5101  TGGAGGGGAA TCCTATCTGT GTTCAAATTC CGTGGGATAA GAATCCTGAG
    A  L  A  K  W  A  E  G  R  T  G  F  P  W  I  D  A·
5151  GCTTTGGCCA AATGGGCAGA AGGCAGGACA GGTTTTCCTT GGATTGATGC
   ·I  M  T  Q  L  R  Q  E  G  W  I  H  H  L  A  R  H·
5201  AATTATGACA CAACTTCGTC AGGAAGGTTG GATTCACCAT TTAGCGCGGC
   ·  A  V  A  C  F  L  T  R  G  D  L  W  I  S  W  E
5251  ATGCTGTAGC ATGCTTTCTG ACTCGAGGTG ACCTTTGGAT TAGCTGGGAA
    E  G  M  K  V  F  E  E  L  L  L  D  A  D  W  S  V·
5301  GAAGGAATGA AGGTCTTTGA AGAGCTGTTA CTTGATGCAG ATTGGAGTGT
   ·N  A  G  S  W  M  W  L  S  C  S  S  F  F  Q  Q  F·
5351  GAATGCTGGA AGCTGGATGT GGCTATCCTG TAGTTCCTTC TTTCAGCAGT
   ·  F  H  C  Y  C  P  V  G  F  G  R  R  T  D  P  N
5401  TTTTTCACTG CTACTGCCCA GTGGGTTTTG GCAGAAGAAC TGACCCAAAT
    G  D  Y  I  R  R  Y  L  P  V  L  R  G  F  P  A  K·
5451  GGGGATTATA TCAGACGTTA TTTACCAGTA CTTAGAGGTT TCCCTGCAAA
   ·Y  I  Y  D  P  W  N  A  P  E  S  I  Q  K  A  A  K·
5501  ATACATCTAT GATCCTTGGA ATGCCCCAGA GAGCATCCAG AAGGCTGCAA
   ·  C  I  I  G  V  N  Y  P  K  P  M  V  N  H  A  E
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    A  S  R  L  N  I  E  R  M  K  Q  I  Y  Q  Q  L  S·
5601  GCAAGCCGTC TGAATATTGA GAGGATGAAA CAGATCTACC AGCAGCTTTC
   ·R  Y  R  G  L  G  L  L  A  T  *  *
5651  ACGATACAGA GGACTGGGTC TTCTTGCAAC TTGATAA
```

Protein data

NtermHis-CRY1-PHR

Plasmid number **#874, #875, #876, #877**

Plasmid name **pDIA92B-NtermHis-CRY1PHR**

Amino acid sequence **510 aa**

analysis	*gw*Cry1-PHR
Length	510 aa
Molecular Weight	58852.73 m.w.
1 µg =	16.992 pMoles
Molar Extinction coefficient	121210
1 A[280] corr. to	0.49 mg/ml
A[280] of 1 mg/ml	2.06 AU
Isoelectric Point	8.48
Charge at pH 7	6.11

amino acid(s)	number count	% by weight	% by frequency
Charged (RKHYCDE)	167	37.63	32.75
Acidic (DE)	58	12.03	11.37
Basic (KR)	63	15.02	12.35
Polar (NCQSTY)	114	22.10	22.35
Hydrophobic (AILFWV)	183	35.62	35.88

amino acid(s)	number count	% by weight	% by frequency
A Ala	36	4.71	7.06
C Cys	12	2.14	2.35
D Asp	25	4.89	4.90
E Glu	33	7.14	6.47
F Phe	25	6.07	4.90
G Gly	32	3.53	6.27
H His	16	3.65	3.14
I Ile	26	5.01	5.10
K Lys	27	5.80	5.29
L Leu	54	10.41	10.59
M Met	12	2.63	2.35
N Asn	22	4.27	4.31
P Pro	32	5.42	6.27
Q Gln	15	3.22	2.94
R Arg	36	9.22	7.06
S Ser	27	4.17	5.29
T Thr	20	3.50	3.92
V Val	25	4.31	4.90
W Trp	17	5.10	3.33
Y Tyr	18	4.79	3.53
B Asx	47	9.16	9.22
Z Glx	48	10.36	9.41
X Xxx	0	0.00	0.00

Aminoacid sequence:

MHHHHHHAMGVNAVHWFRKGLRLHDNPALRECIQGADT
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LNSRLFVIRGQPADVFPRLFKEWNIAKLSIEYDSEPFG
KERDAAIKKLASEAGVEVIVRISHTLYDLDKIIELNGG
QPPLTYKRFQTLISRMEPLEMPVETITPEVMKKCTTPV
SDDHDEKYGVPSLEELGFDTDGLPSAVWPGGETEALTR
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CLSCRLFYFKLTDLYKKVKKNSSPPLSLYGQLLWREFF
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SWEEGMKVFEELLLDADWSVNAGSWMWLSCSSFFQQFF
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NAPESIQKAAKCIIGVNYPKPMVNHAEASRLNIERMKQ
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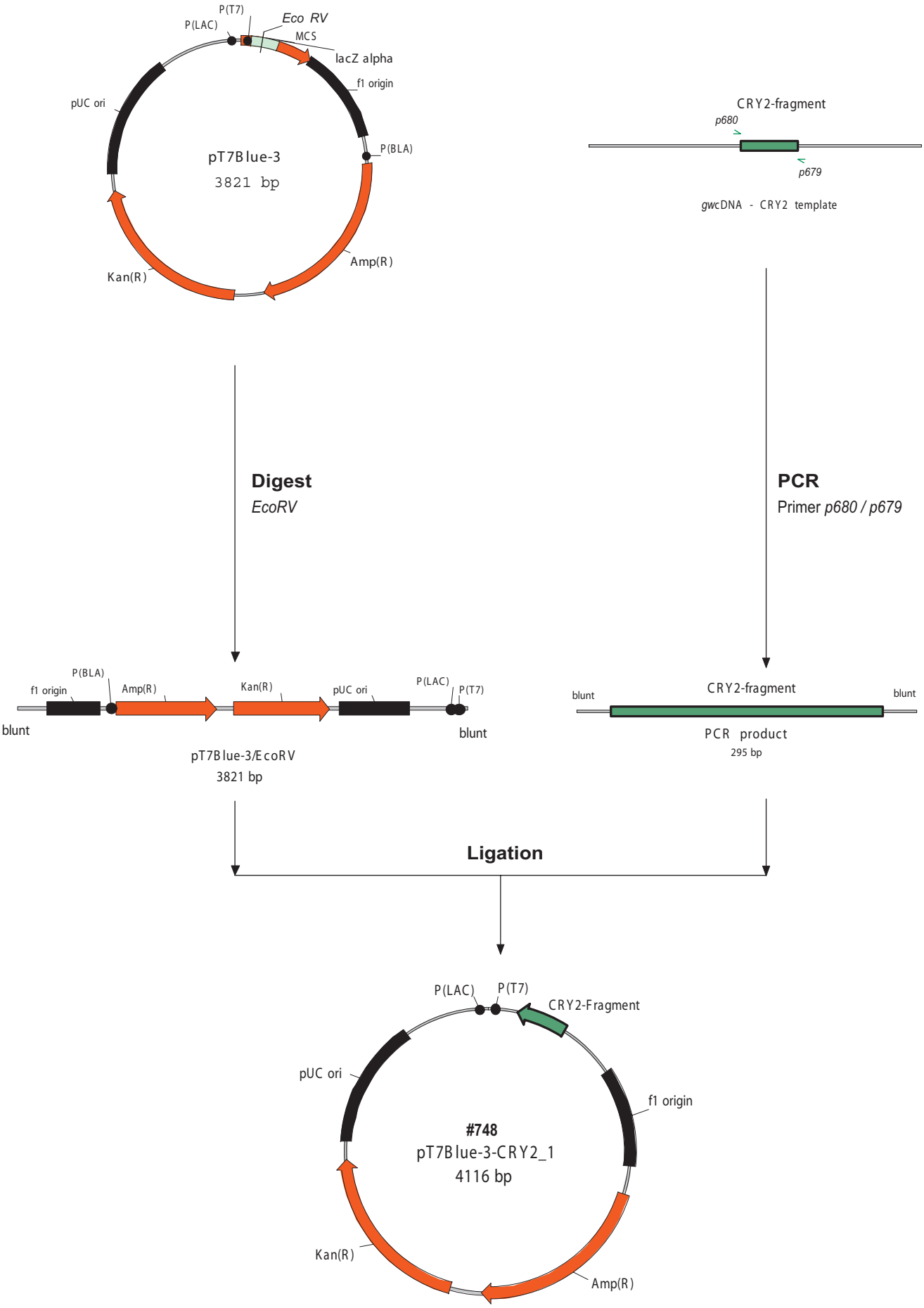
***gw*CRY2** – cloning strategy & sequence data

[GenBank accession no. AY739908]



Supplementary **Figure 9: Identification and cloning strategy of a garden warbler cryptochrome 2 fragment, *gw*Cry2 (plasmid #748)** The PCR-amplified (primer pair *p679* & *p680*) PCR product was cloned into pTBlue-3 by means of the Perfectly Blunt cloning kit (Novagen) and transformed into NovaBlue cells. Theoretical reconstruction of the cloning strategy was performed by means of *VectorNTI™* (Invitrogen). For abbreviations see *Supplementary* Figure 3.

Sequence data

*gw*CRY2 fragment #748

Plasmid number	**#748**
Plasmid name	**pT7Blue-3-CRY2_1**
Nucleic acid sequence	**273 bp**

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051     AGACAGGCTT CCCTTGGATT GATGCAATCA TGACCCAACT GAGACAAGAG

101     GGGTGGATCC ACCATCTGGC CAGGCATGCA GTGGCCTGCT TCCTGACCAG

151     GGGTGACCTC TGGATCAGCT GGGAGTCAGG AGTCAGGGTG TTTGACGAAC

201     TGTTGCTGGA TGCAGATTTC AGCGTGAATG CAGGCAG
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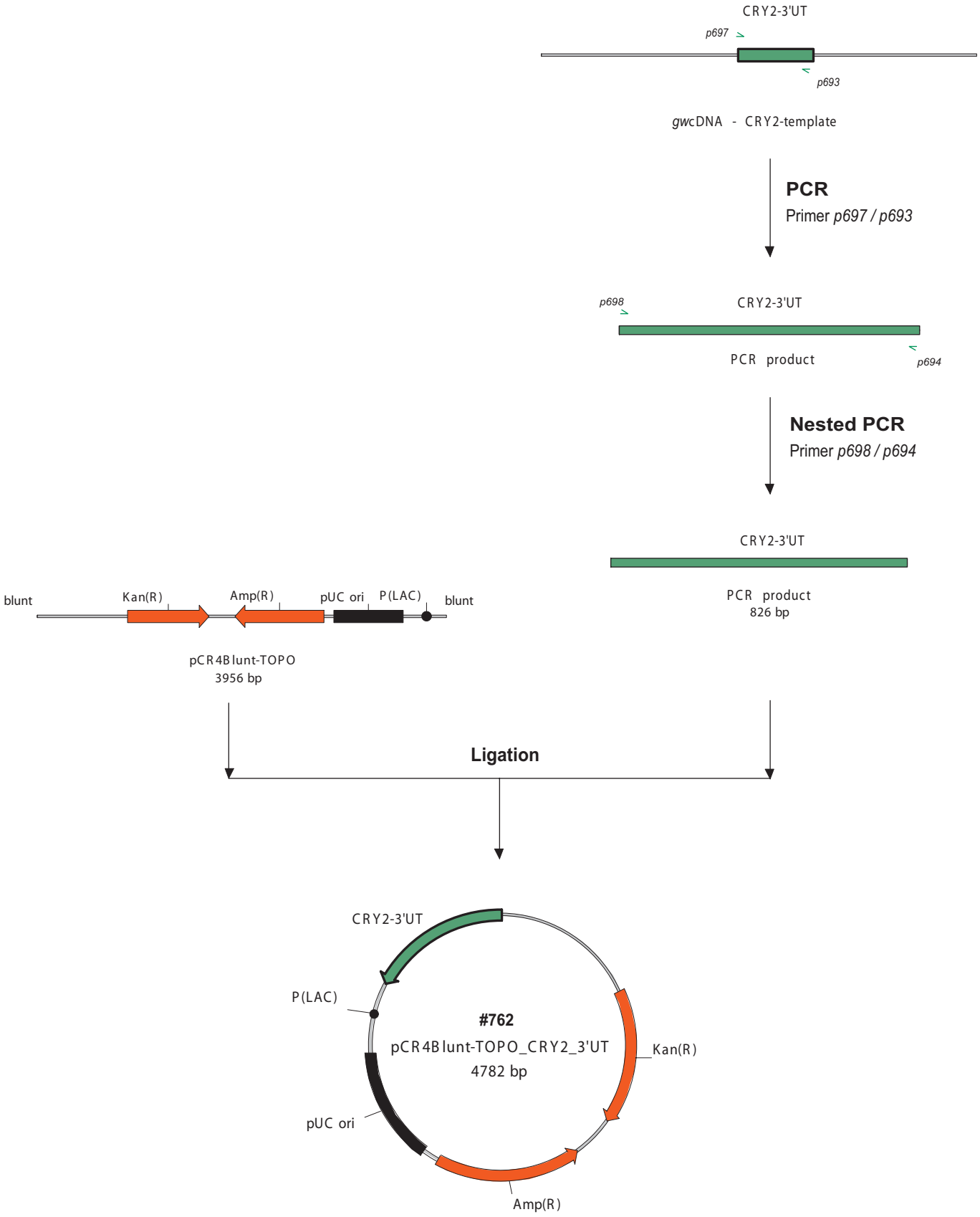

Supplementary **Figure 10: Cloning strategy of the 3' region of garden warbler cryptochrome 2, *gw*Cry2 (plasmid #762)** Based on the internal *gw*Cry2 sequence from clone #748 (see *Suppl. I* Figure 9), the 3'-part of garden warbler Cry2 cDNA was amplified in a nested PCR (primer combinations: *p697/p693* and *p698/p694*). The derived 3' *gw*Cry2 sequence is highlighted in green. Theoretical reconstruction of the cloning strategy was performed by means of *VectorNTI™* (Invitrogen). For abbreviations see *Supplementary* Figure 3.

Sequence data

*gw*CRY2 fragment #762

Plasmid number	**#762**
Plasmid name	**pCR4Blunt-TOPO_CRY2_3'UT**
Nucleic acid sequence	**732 bp**
Amino acid sequence	**244 as**

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         · L  R  Q   E  G  W  I   H  H  L   A  R  H   A  V  A  C ·
051      ACTGAGACAA GAGGGGTGGA TCCACCATCT GGCCAGGCAT GCAGTGGCCT

         · F  L  T   R  G  D  L   W  I  S   W  E  S   G  V  R
101      GCTTCCTGAC CAGGGGTGAC CTCTGGATCA GCTGGGAGTC AGGAGTCAGG

          V  F  D  E   L  L  L   D  A  D   F  S  V  N   A  G  S ·
151      GTGTTTGACG AACTGTTGCT GGATGCAGAT TTCAGCGTGA ATGCAGGCAG

         · W  M  W   L  S  C  S   A  F  F   Q  Q  F   F  H  C  Y ·
201      CTGGATGTGG CTCTCGTGCA GTGCGTTCTT CCAACAGTTC TTCCACTGTT

         · C  P  V   G  F  G   R  R  T  D   P  S  G   D  Y  V
251      ACTGTCCTGT GGGCTTCGGA CGTCGCACGG ACCCCAGCGG TGACTATGTG

          K  R  Y  L   P  K  L   K  G  F   P  S  R  Y   I  Y  E ·
301      AAGAGGTATC TGCCCAAACT AAAGGGTTTC CCCTCACGAT ATATCTACGA

         · P  W  N   A  P  E  S   V  Q  K   A  A  K   C  I  I  G ·
351      ACCATGGAAT GCCCCAGAGT CTGTGCAGAA GGCAGCCAAG TGCATCATTG

         · V  D  Y   P  K  P   M  V  N  H   A  E  T   S  R  L
401      GGGTGGACTA CCCCAAACCC ATGGTGAACC ATGCAGAGAC CAGCCGACTG

          N  I  E  R   M  K  Q   I  Y  Q   Q  L  S  R   Y  R  G ·
451      AACATCGAGC GCATGAAGCA GATCTACCAG CAGCTGTCAC GCTATAGGGG

         · L  C  L   L  A  S  V   P  S  C   V  E  D   L  S  S  P ·
501      CCTCTGTTTG CTGGCATCTG TCCCTTCGTG TGTGGAAGAT CTCAGTAGCC

         · V  T  D   S  A  S   G  P  G  C   S  T  S   T  A  V
551      CAGTCACAGA CTCAGCTTCA GGGCCGGGCT GCAGCACCAG CACAGCAGTG

          R  L  S  Q   A  D  Q   A  S  P   K  R  K  H   E  G  A ·
601      AGGCTGTCTC AAGCAGACCA GGCTTCTCCA AAACGCAAAC ATGAGGGAGC

         · E  E  P   C  P  E  E   L  Y  K   R  A  K   V  T  G  L ·
651      GGAAGAGCCG TGCCCTGAAG AACTGTACAA ACGAGCCAAA GTGACAGGTC

         · P  A  S   E  I  P   G  K  S  L
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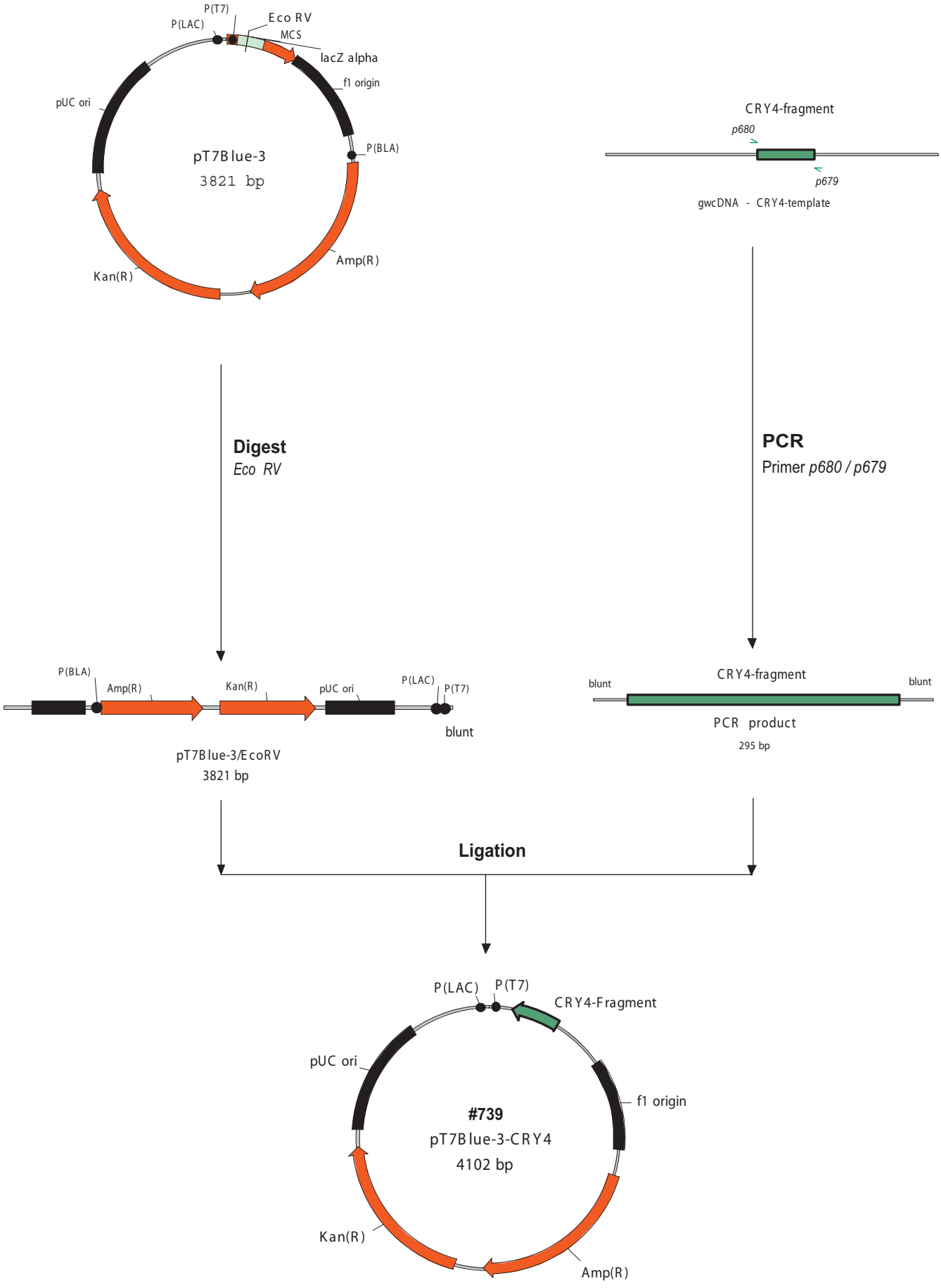

Supplementary **Figure 11: Identification and cloning strategy of a fragment of garden warbler cryptochrome 4, *gw*Cry4 (plasmid #739)** The PCR-amplified (primer pair *p679 & p680*) PCR product was cloned into pTBlue-3 by means of the Perfectly Blunt cloning kit (Novagen) and transformed into NovaBlue cells., similar to plasmid #748 (*gw*Cry2) Theoretical reconstruction of the cloning strategy was performed by means of *VectorNTI™* (Invitrogen). For abbreviations see *Supplementary* Figure 3.

Sequence data

*gw*CRY4 fragment #739

Plasmid number	**#739**
Plasmid name	**pT7Blue-3-CRY2_1 CRY4!**
Nucleic acid sequence	**273 bp**

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051     AGACAGGGTT CCCATGGATT GATGCCATTA TGACCCAGCT GCGCCAGGAA

101     GGTTGGATCC ATCACCTGGC TCGGCATGCT GTCGCCTGCT TCCTGACACG

151     GGGGCACCTT TGGATCAGCT GGGAAGAGGG AATGAAGGTG TTTGAAGAGC

201     TGCTCATAGA TGCTGACTAT AGCATCAATG CTGGGAA
```

Paper IV

Mouritsen, H., Feenders, G., Liedvogel, M., Wada, K. & Jarvis, E.D.:

Night-vision brain area in migratory songbirds.

PNAS **102**, 8339-8344, 2005.

Night-vision brain area in migratory songbirds

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Edited by Marcus E. Raichle, Washington University School of Medicine, St. Louis, MO, and approved April 15, 2005 (received for review December 21, 2004)

Twice each year, millions of night-migratory songbirds migrate thousands of kilometers. To find their way, they must process and integrate spatiotemporal information from a variety of cues including the Earth's magnetic field and the night-time starry sky. By using sensory-driven gene expression, we discovered that night-migratory songbirds possess a tight cluster of brain regions highly active only during night vision. This cluster, here named ''cluster N,'' is located at the dorsal surface of the brain and is adjacent to a known visual pathway. In contrast, neuronal activation of cluster N was not increased in nonmigratory birds during the night, and it disappeared in migrants when both eyes were covered. We suggest that in night-migratory songbirds cluster N is involved in enhanced night vision, and that it could be integrating vision-mediated magnetic and/or star compass information for night-time navigation. Our findings thus represent an anatomical and functional demonstration of a specific night-vision brain area.

behavioral molecular mapping | bird orientation | cognition | magnetic sense | ZENK (zif268, Egr-1, NGF-1A, and Krox-24)

Night-migratory songbirds use both a geomagnetic and a star compass to orient during migration (1–6) but the underlying brain circuits are unknown. Star-compass orientation requires vision in dim light for processing constellations of the night-time starry sky. Surprisingly, magnetic-compass orientation also seems to require night vision: magnetic-compass orientation is dependent on the wavelength of the dim ambient light (4, 7), birds with their right eye covered seem unable to perform magnetic compass orientation (8), and birds can still perform magnetic compass orientation with their pineal gland removed (9). Current evidence suggests that visual sensing of the magnetic field occurs through the eyes by means of a light-activated, radical-pair based magnetodetector (4, 7, 10–13). Another type of magnetodetector based on magnetite seems to be predominantly involved in detecting changes in magnetic intensity and/or inclination (4, 14–16). Together, these findings predict that processing of both magnetic- and star-compass information during night-time migration requires specialized night-time visual processing in night-migratory songbirds. We tested this hypothesis by using sensory-driven gene expression (17) to identify brain regions involved in night- and day-time vision in migratory and nonmigratory songbirds. Our results suggest that night-migratory songbirds do indeed possess a brain area specialized for night vision.

Methods

Test Subjects. We examined two distantly related species of wild-caught night-migratory songbirds [garden warblers (GWs), *Sylvia borin*; and European robins (ERs), *Erithacus rubecula*] and two distantly related species of nonmigratory songbirds [zebra finches (ZFs), *Taeniopygia guttata*; and canaries (CNs), *Serinus canaria*]. The GWs, ERs, and ZFs were kept inside a wooden building under the local photoperiod for at least 5 days before the experiment. Behavioral tests were performed during the GW and ER migratory seasons from August 22 to October 18, 2002 and 2003 and April 15 to May 25, 2003, except for three GWs tested July 17 to August 3, 2003. The canaries were taken from the Jarvis laboratory collection of birds when awake at night and day to confirm consistency between nonmigrants. All animal procedures were approved by the Animal Care and Use Committees of Bezirksregierung Weser-Ems (Oldenburg, Germany) and/or Duke University Medical Center (Durham, NC).

Test Procedures. On testing day, our night-group birds (n = 12 GWs, 4 ERs, and 5 ZFs) were individually put into a custom-designed cylindrical, transparent Plexiglas cage fitted with a circular perch placed 8.5 cm above the ground in the center of the cage (18) (the two CNs were placed in sound isolation boxes). The birds were allowed to get used to the cage or soundbox for 3–12 h. At dusk (local photoperiod), the room lights were turned off except for four small, diffused light bulbs simulating a natural moonlit night (0.04 lux). This light intensity is typically used in behavioral orientation tests with night-migrants (2, 6, 7, 10). Our day-group birds (n = 5 GWs, 5 ZFs, and 5 CNs) were tested in full room light (≈275 lux). Each bird's behavior was continuously observed in real time by two infrared (840 nm) video cameras (top-view and side-view) connected to a split-screen surveillance monitor and in parallel recorded to videotape (25 frames per sec). No night-bird was collected earlier than 100 min after the lights went off, thereby ensuring that any possible brain activity induced by the day/night transition had decreased to its baseline level by the time the bird was collected. We also took care that no external acoustic signals reached the bird. To minimize brain activity because of movement and other factors, we collected birds only after they had been sitting relatively still but awake (eyes open) for at least 45 min but mostly for 60 min or more, while a minimum of other behaviors occurred (0–16 flights [most performed 0 flights] and occasional walking on the perch or floor; exception: ZFs tested during the day did not sit still). After the birds were killed, their brains were rapidly dissected, embedded in Tissue-Tek O.C.T. (Sakura Finetek, Zoeterwoude, The Netherlands), and quick-frozen in a dry ice/ethanol bath at −80°C.

Eye Capping. The ERs (n = 4) night-time group with eyes open was compared with another group of ERs (n = 4) fitted with light-tight eye caps to prevent any visual stimulus from reaching the eyes. The eye caps were built of small black plastic–velour cylinders (diameter = 10 mm) enlarged with Leucoplast tape (Beiersdorf AG, Hamburg, Germany) and light-tight black tape. Three to 5 h before onset of darkness, the eye caps were glued onto the bird's head to cover the eyes completely without any direct contact to the eyes themselves. The bird was put into the cage, observed continuously, and collected as described.

Gene Expression Analyses. We measured expression of ZENK [acronym for zif268, Egr-1, NGF-1A, and Krox-24 (19)] and c-fos immediate early genes in the brain. Expression of ZENK and c-fos mRNA in the brain is driven by neuronal activity and can

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: GW, garden warbler (*Sylvia borin*); ER, European robin (*Erithacus rubecula*); ZF, zebra finch (*Taeniopygia guttata*); CN, canary (*Serinus canaria*); ZENK, zif268, Egr-1, NGF-1A, and Krox-24; MD, dorsal mesopallium; DNH, dorsal nucleus of the hyperpallium; GluR1, glutamate receptor 1 subunit.

†To whom correspondence may be addressed. E-mail: henrik.mouritsen@uni-oldenburg.de or jarvis@neuro.duke.edu.

be detected in neurons ≈5 min after onset of neural firing with peak expression after 30–45 min (17, 20). Therefore, increased cumulative mRNA expression marks brain regions that were active during the last 45–60 min of sensory stimulation or behavior. However, ZENK and c-fos are not activated by neural activity in all brain cell types. These exceptions in birds are some thalamic neurons, telencephalic neurons receiving primary sensory input from the thalamus, and globus pallidus neurons (21). It has been suggested that these neurons do not express the specific neurotransmitter receptors necessary to induce ZENK and c-fos expression in response to neuronal activity (20). In all other neuron types, which constitute roughly two-thirds of the avian brain, expression of ZENK and/or c-fos follows neuronal firing.

For one bird per group, 12-μm frozen sections were cut throughout the entire brain (left hemisphere in the sagittal plane and the right hemisphere in the coronal plane). We observed no differences in gene expression between left and right hemispheres and therefore continued cutting only sagittal sections of the left hemisphere for all remaining birds. We chose sagittal sections because cluster N was best seen in this plane. Corresponding sections of all birds were fixed in 4% paraformaldehyde and processed by *in situ* hybridization with antisense 5′-[α-[^{35}S]thio]UTP-labeled riboprobes of a ZF ZENK (K.W. and E.D.J. clone), c-fos (K.W. and E.D.J., unpublished data), or glutamate receptor 1 subunit (GluR1) of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor cDNAs (22) by following described procedures (22). We used GluR1 as an anatomical marker in combination with Nissl staining to identify anatomical boundaries of cerebral subdivisions. The hybridized sections were first exposed to x-ray films (Biomax MR, Kodak) for 1–4 days, dipped into an autoradiographic emulsion (NTB2, Kodak), incubated for 3–4 weeks at 4°C, processed with Kodak developer (D-19) and fixer, and Nissl-stained with cresyl violet acetate solution (Sigma). X-ray film brain images were digitally scanned from a dissecting microscope connected to a SPOT III charge-coupled device camera with SPOTadvanced imaging software. For quantification, a person naïve to the experimental conditions used PHOTOSHOP 6.0 (Adobe Systems, San Jose, CA) to measure the mean pixel intensities in the brain regions of interest on a 256-level gray scale pixel range. We measured ZENK expression by calculating the difference in mean pixel density between the relevant brain region and a comparably sized region anterior to it in the same brain subdivision(s) [e.g., cluster N − (anterior hyperpallium + anterior dorsal mesopallium)]. We use the term "region" to refer to a specific part of a brain subdivision and the term "area" to refer to a cluster of regions. To test for significant differences between individual groups, the SIGMASTAT software package was used to perform one-way ANOVA followed by a Student–Newman–Keuls multicomparison.

Results

Both species of night-migratory songbirds when awake at night showed a striking pattern of high ZENK expression confined to the dorsal part of the cerebrum (Fig. 1*A* II, yellow arrow). ZENK expression in this area was not affected by movement behavior (unpublished work) and only medium to low levels of ZENK expression were found throughout the rest of the brain. To anatomically define this cerebral area showing very high expression, in addition to examining Nissl stains, we hybridized adjacent brain sections to GluR1, which distinguishes the boundaries between the avian cerebral brain subdivisions (22, 23). The GluR1- and Nissl-stained pattern revealed that this area consists of five brain regions (Fig. 1*B*): (*i*) a portion of the hyperpallium apicale, (*ii*) a portion of the interstitial region of the hyperpallium apicale, (*iii*) a portion of the dorsal mesopallium (*iv*) a nucleus embedded within this portion of the hyperpallium apicale that we named dorsal nucleus of the hyperpallium (DNH), and (*v*) a shell of cells around DNH that we named the DNH-shell. We designated these regions according to the recent anatomical nomenclature of the avian brain (23). The DNH had higher GluR1 and lower ZENK expression relative to the surrounding hyperpallium, indicating that it behaves differently from the surrounding hyperpallium. The DNH-shell had slightly lower ZENK expression and was characteristically different from the other parts of the hyperpallium (Fig. 1*B*; and Dominik Heyers, personal communication). In contrast to the strong night-time activation, this cluster of regions showed no significant ZENK induction during the day (Fig. 1 *A*I and *C*). c-fos, another activity-dependent gene, like ZENK, also showed strikingly high expression in this cluster of regions at night (Fig. 2). As expected, GluR1 did not show a difference between day and night (data not shown). We therefore named this group of five regions "cluster N" (N for night-activation). Cluster N is fairly large, taking up ≈40% of the hyperpallium and dorsal mesopallium in the sagittal slice where cluster N activation is most prominent. It extends ≈1 mm rostrocaudal, ≈1.5 mm mediolateral, and ≈1.5 mm dorsoventral.

The strong night-time activation in cluster N observed in the two migratory songbird species was not found in the two nonmigratory species (Fig. 1 *A* and *C*). Although, in nonmigrants, expression in the entire hyperpallium and MD (posterior to anterior) in the equivalent sagittal plane was slightly higher than in other brain regions at night, there was a decreasing tendency in absolute ZENK expression levels at night [mean intensity index: nonmigrants day = 95 ± 13 (SD), nonmigrants night = 76 ± 20; *t* test $P = 0.15$].

The hyperpallium is also known as the "Wulst" (meaning bulge) with its central–dorsal part functioning as the "visual Wulst" (24). Because no defined anatomical boundaries are known of the visual Wulst, we cannot prove whether cluster N is a part of the visual Wulst (being either a specialization or having evolved out of it) or whether it is located adjacent to it. However, a relationship to the visual Wulst is very likely because the anterior part of cluster N is localized in the mediodorsal part of the hyperpallium, which corresponds to the described posterior extensions of the visual Wulst (24). Based on this knowledge and on the predicted visual nature of magnetic- and star-compass input to the brain in combination with the fact that cluster N activation seems specific to night-migrants, we suspected that cluster N could be processing visual information at night. To test this hypothesis, we performed ZENK *in situ* hybridization on brains from night-migrants that had both eyes covered by light-tight eye caps during the dim-light night. After becoming used to the eye caps, these birds sat relatively still for extended periods of time, as did the birds without eye cover. Blocking visual input resulted in strong reduction of dim-light, night-time-induced cluster N ZENK expression (Fig. 3 *A*–*C*). The DNH-shell did not show reduction of ZENK expression, and thus these cells are still active in complete darkness (Fig. 3*B*). We conclude that the majority of cluster N activation requires dim-light, visual input.

To check for possible circadian effects, we compared cluster N expression levels in birds at multiple time points after the onset of night-time, dim-light levels (within a 6-h range) and found that the expression levels in cluster N in birds with both eyes open or closed did not correlate with the time of brain collection relative to the onset of dim-light levels (Fig. 3*D*). Furthermore, birds with covered eyes showed low ZENK expression at times when other birds sitting in dim-light show high ZENK expression in cluster N (Fig. 3*D*). Thus, cluster N activation cannot simply be because of circadian rhythms.

To test for a possible seasonal variable, we examined three GWs during their nonmigratory breeding season in July and early August, when they did not show evidence of migratory

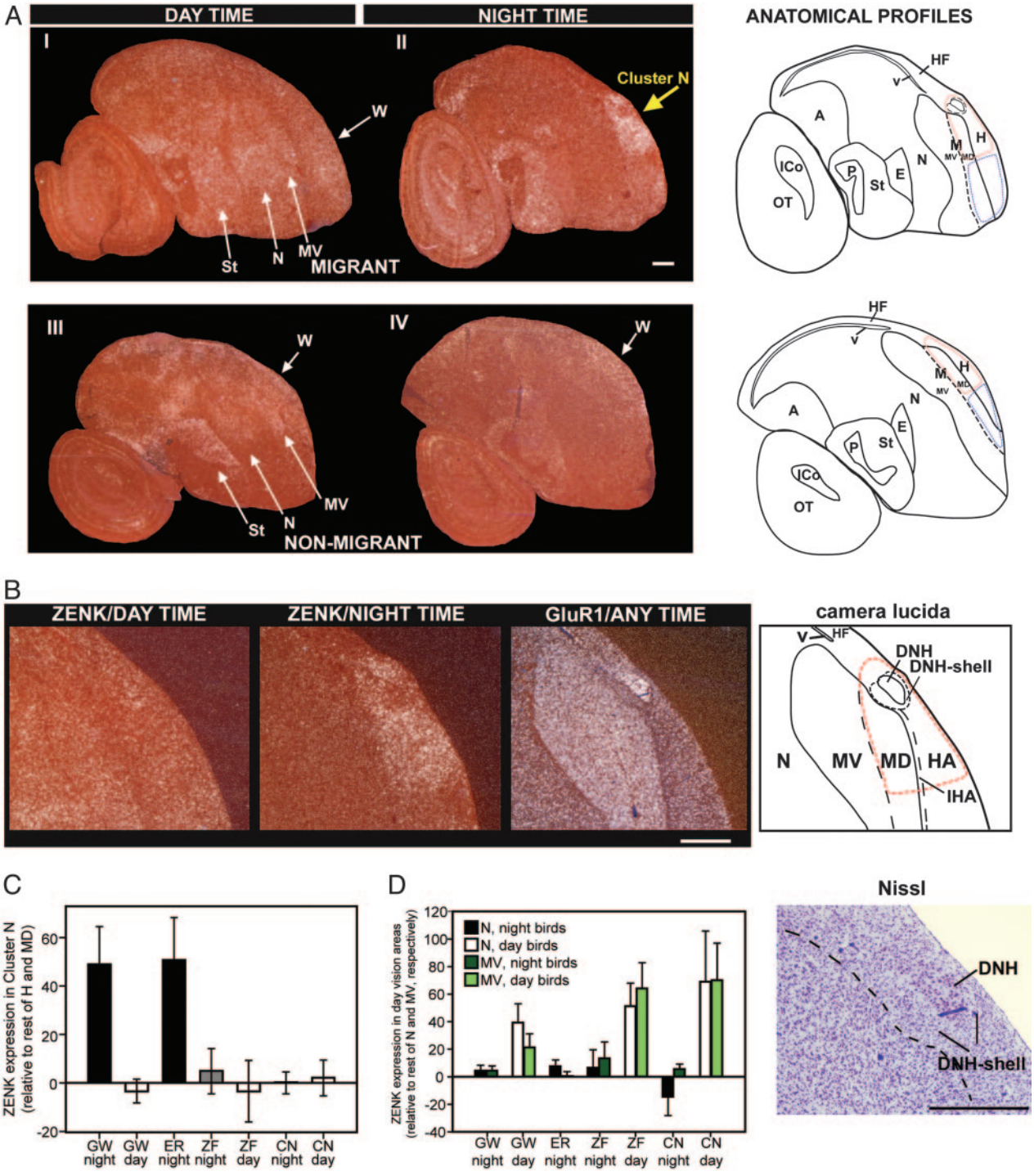

Fig. 1. ZENK brain activation patterns. (*A*) Day- and night-time visual activation in night-migratory GWs (I and II) and nonmigratory ZFs (III and IV). Parasagittal brain sections are shown. (Dorsal is up; anterior is right; white-silver grains, ZENK expression; red, Nissl stain; white arrows in I and III, regions activated during day-time vision; yellow arrow in II, regions activated in migrants during night-vision.) Arrows are drawn at different angles for each species to reflect that the relative orientation of the GW cerebrum is naturally angled more forward than the ZF cerebrum. The red and blue highlighted regions in the anatomical drawing indicate the regions quantified from for *C*. (*B*) Anatomical characterization of cluster N in GWs. The red-dashed line highlights the cluster N area of ZENK expression. (*C*) Differences in cluster N ZENK expression among groups were highly significant {cluster N, i.e., [posterior hyperpallium (H) + dorsal mesopallium (MD)] − [anterior H + MD]; by one-way ANOVA, df = 6; $df_{residual}$ = 29; $F = 22.4$; $P < 0.001$}. Relative cluster N ZENK expression in GWs and ERs at night was significantly higher compared with all other groups (Student–Newman–Keuls multicomparison test: $P < 0.001$ for all individual comparisons). Relative cluster N ZENK expression in GWs during the day and in the comparable region in nonmigrants (ZFs; CNs) during the day or night was not significantly different among each other (Student–Newman–Keuls multicomparison test: $P > 0.73$ for all comparisons) nor from anterior H + MD (all 95% confidence intervals include 0, which indicates identical expression in cluster N and the rest of H + MD.) The *y* axis shows relative pixel density on a 256 gray scale. (*D*) ZENK induction in known day-vision regions was significantly higher during the day (one-way ANOVA: $P < 0.001$ for all within-species comparisons between day and night birds except the mesopallium ventrale (MV) comparison for GWs where $P = 0.06$). Error bars = standard deviations. A, arcopallium; P, pallidum; E, entopallium; St, striatum; N, nidopallium; M, mesopallium; H, hyperpallium; ICo, intercollicular complex; v, ventricle; OT, optic tectum; HF, hippocampal formation; IHA, interstitial region of the hyperpallium apicale (HA); DNH, dorsal nucleus of the hyperpallium; W, visual Wulst). (Scale bars, 0.5 mm.) A high-resolution PDF version of Fig. 1 is available in the supporting information on the PNAS web site.

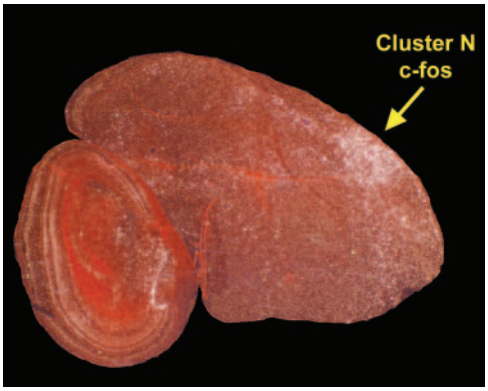

Fig. 2. Example of cluster N c-fos induction at night in night-migrants (GW). The sagittal section is more lateral than that in Fig. 1, where the DNH nucleus is no longer present. A high-resolution PDF version of Fig. 2 is available in the supporting information on the PNAS web site.

activity behavior at night. These animals showed increased ZENK expression in cluster N during dim-light, night-time conditions and at expression levels that did not differ from those seen in birds tested during the migration season (*t* test, $P = 0.37$). Thus, cluster N night-time activation is not purely due to the increase of night-time activity during the migratory season in the migrants but appears to be specific to night-migratory species at all times of the year.

In contrast to the night-time activation in cluster N, during day-light hours consistent increases in expression occurred in a set of regions surrounding the entopallium in both night-migratory and nonmigratory birds (Fig. 1 *A*I, 1*A*III, and 1*D*). The entopallium receives thalamic visual input, and like other primary sensory telencephalic neurons it does not show prominent ZENK expression (21). The day-time activated regions around the entopallium included a portion of the nidopallium and a portion of the ventral mesopallium, forming a ventral to dorsal column of brain activation. We suggest that these regions are involved in day-time vision. Support for this suggestion comes from visual pathway connectivity and vision-activated electrophysiological neural firing in these brain regions in ZFs and other bird species (25, 26).

Discussion

Our results identify a brain area, cluster N, active specifically in night-vision. We suggest that cluster N is evolutionarily related to the visual Wulst. It appears to be localized either posterior to or as a specialized part of the visual Wulst, a visual area comparable to the mammalian striate visual cortex V1 (27). The visual Wulst is part of the thalamofugal pathway (striate visual

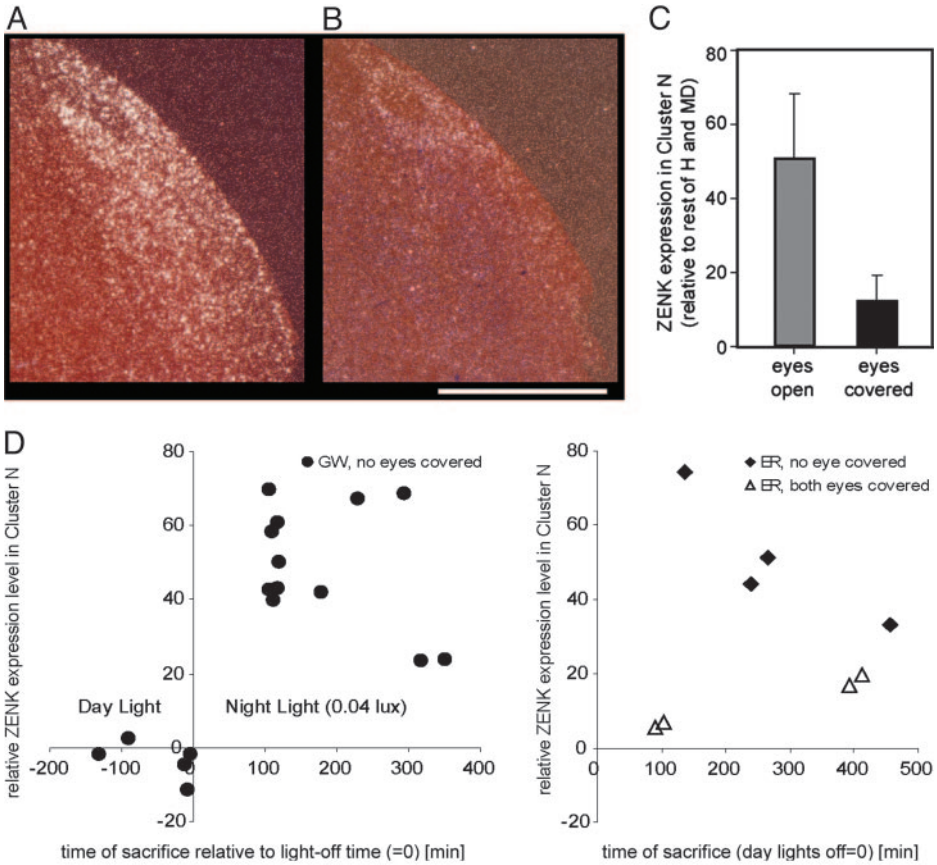

Fig. 3. Cluster N ZENK induction at night in night-migrants is visually driven. (*A* and *B*) ZENK expression in cluster N in a night-migrant, ER, with eyes open (*A*) or with both eyes covered by light-tight eye-caps (*B*). (Scale bar, 1 mm.) (*C*) Covering the birds' eyes led to a significant reduction in cluster N ZENK expression (*t* test, $t = 4.080$; df = 6; $P < 0.01$; $n = 4$ per group). The expression in the covered-eye birds is still above zero because the cluster N area quantified included the shell of high expression still present around the DNH nucleus (refer to *B*). (*D*) Cluster N ZENK expression as a function of time for all migratory birds used in this study. (*Left*) GWs with both eyes open. Along the *x* axis, time point 0 is when the day-lights were turned off and the dim night-time lights were turned on. The amount of time the lights were off did not affect increased cluster N ZENK expression (linear regression of night-time values, $F = 1.55$, $P = 0.24$). (*Right*) Birds with covered eyes show low ZENK expression at times when other birds sitting in dim-light show high ZENK expression throughout cluster N. A high-resolution PDF version of Fig. 3 is available in the supporting information on the PNAS web site.

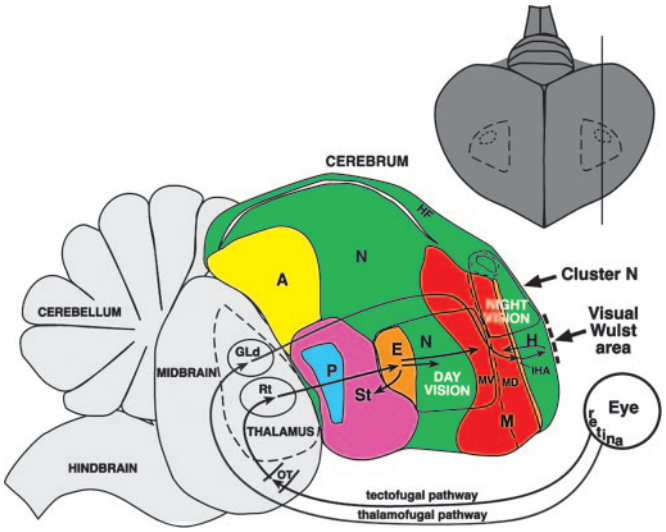

Fig. 4. Schematic drawing of a brain showing the relative locations of the day- and night-vision (cluster N) activated brain regions in night-migrant songbirds. The thamalofugal and tectofugal visual pathways have been determined in other bird species. Upper right, the extent of cluster N seen from the dorsal surface of the brain, determined from serial parasagittal and coronal sections hybridized to ZENK. GLd, lateral geniculate nucleus, dorsal part; Rt, nucleus rotundus; additional abbreviations are as in legend of Fig. 1.

pathway in mammals) that transfers information directly from the retina to the thalamus, then to the interstitial region of the hyperpallium apicale, and finally to hyperpallium apicale (Fig. 4). The thalamofugal pathway is separate from another visual system called the tectofugal pathway (extrastriate visual pathway in mammals). The avian tectofugal pathway transfers information from the retina to the tectum of the midbrain and, from there, via the thalamus to the entopallium, surrounding nidopallium, and ventral mesopallium (Fig. 4) (25). In nonmigrants, we found that both cerebral visual pathways showed day-time activation. At night, only the visual Wulst area of the thalamofugal pathway still showed some activation, albeit decreased in relation to the day-time level. However, in migrants cluster N showed very high activation at night. These findings cannot prove whether nonmigrants actually do not possess an area comparable to cluster N or whether they do have such an area that does not show enhanced activation during the night. However, because the visual part of the Wulst is typically more centrally located (posterior–anterior) in the hyperpallium, and cluster N is located at the posterior end of hyperpallium, the results suggest that in night-migratory birds, cluster N evolved out of the preexisting thalamofugal visual pathway as a specialized posterior part of or an attachment to the visual Wulst.

The pattern of night-time and day-time activation suggests another view of the functional organization of the avian cerebrum (Fig. 4). The dorsal mesopallium and the hyperpallium above it are activated as a columnar unit (i.e., cluster N), whereas the ventral mesopallium and the nidopallium ventral to it are activated as another columnar unit (day-time visual activation). This pattern of activation suggests that the hyperpallium is functionally associated with the dorsal mesopallium, whereas the nidopallium is functionally associated with the ventral mesopallium, and that perhaps cluster N with its five subregions represents a functional cerebral system.

The increased ZENK and c-fos expression in cluster N and the fact that this increased expression disappears when the birds' eyes were covered suggest that cluster N increased neural firing during night vision. The consequence of induction of ZENK and other immediate early genes in cluster N of night-migratory songbirds is presumably the same as that proposed for other brain areas that express ZENK (17, 20) (i.e., it regulates the transcription of other genes to either replace proteins that get metabolized when the involved neurons become highly active or to stabilize circuits involved in new information processing).

Why would night-migrants need to evolve a distinct night-vision system, when all songbird species are able to see at night? We suggest that night-migrants may require specialized development of a cerebral system such as cluster N for seeing better at night and/or for visual night-time navigation. The navigation signals sensed and the information processed could be star-light constellations and/or the Earth's magnetic field. The latter possibility would be in line with theoretical (11), molecular (13), and behavioral (4, 7, 8, 10, 12, 18) evidence suggesting that the Earth's magnetic field modulates the light sensitivity of specialized receptor molecules differently in various parts of the retina, leading to perception of the magnetic field as visual patterns (11, 13). As a consequence, the brain regions ultimately extracting the reference direction provided by the geomagnetic field should process and compare purely visual input from the retina. This prediction is in line with ZENK and c-fos expression patterns in the eyes of migratory GWs. Retinal ganglion cells showed high neuronal activity levels at night during magnetic orientation, and these active cells contained high concentrations of cryptochromes, a class of photoreceptor molecules suggested to be involved in magnetodetection (13). In contrast, in nonmigratory ZFs retinal ganglion cells showed lower ZENK expression at night when very little cryptochrome was found in the eyes (13).

The discovery of a distinct night-vision brain area in night-migratory songbirds will allow investigators to probe a specific site in the central nervous system of vertebrates for biological mechanisms of night vision and navigation. Verifying these suggestions will require future studies with electrophysiological recordings and lesions of cluster N.

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Paper V *

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Lateralised activation of Cluster N in the brains of migratory songbirds.

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Lateralised activation of Cluster N in the brains of migratory songbirds

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Abstract

Cluster N is a cluster of forebrain regions found in night-migratory songbirds that shows high activation of activity-dependent gene expression during night-time vision. We have suggested that Cluster N may function as a specialised night-vision area in night-migratory birds and that it may be involved in processing light-mediated magnetic compass information. Here, we investigated these ideas. We found a significant lateralised dominance of Cluster N activation in the right hemisphere of European robins (*Erithacus rubecula*). Activation predominantly originated from the contralateral (left) eye. Garden warblers (*Sylvia borin*) tested under different magnetic field conditions and under monochromatic red light did not show significant differences in Cluster N activation. In the fairly sedentary Sardinian warbler (*Sylvia melanocephala*), which belongs to the same phyolgenetic clade, Cluster N showed prominent activation levels, similar to that observed in garden warblers and European robins. Thus, it seems that Cluster N activation occurs at night in all species within predominantly migratory groups of birds probably because such birds have the capability to switch between migratory and sedentary life styles. The activation studies suggest that although Cluster N is lateralised, as is the dependence on magnetic compass orientation, either Cluster N is not involved in magnetic processing or the magnetic modulations of the primary visual signal – forming the basis for the currently supported light-dependent magnetic compass mechanism – are either relatively small and/or directed in opposite directions such that activity dependent gene expression changes are not sensitive enough to pick them up.

Introduction

Migratory passerines have orientation skills that enable them to travel thousands of kilometres every spring and autumn between their breeding and wintering grounds. Passerines migrating at night can use a geomagnetic compass and/or celestial cues to orient (e.g. Wiltschko, 1967; species overview: Wiltschko & Wiltschko, 1996; Cochran *et al.,* 2004). Despite intensive experimental efforts, the underlying physiological and molecular mechanism(s) of magnetoreception and detection of celestial constellations at night are still not fully understood.

Currently, two hypotheses on how birds may sense the magnetic field are both supported by experimental evidence (review: Mouritsen & Ritz, 2005). One is based on superparamagnetic iron particles in the upper beak region (Fleissner *et al.,* 2003; Mora *et al.,* 2004). Magnetic field distortions have been proposed to affect ion channels leading in turn to a change in membrane potential (Kirschvink *et al.,* 2001). This magnetite-hypothesis is mainly discussed in connection with a magnetic map or signpost sense (e.g. Munro *et al.,* 1997a; Mora *et al.,* 2004). The other hypothesis is based on a magnetic compass sense where the bird extracts global directional information via a light-dependent radical-pair based mechanism (Ritz *et al.,* 2000, 2004). This mechanism requires light input and suggests that light-sensitive molecules in the retina form radical-pairs upon photoexcitation. The direction of the magnetic field relative to the axis of the molecules will modulate the amount of radicals existing in one of two states and thus indirectly affect the light sensitivity of specific chemical reactions (Ritz *et al.,* 2000). Cryptochromes are the most probable candidates as radical-pair forming photoreceptor molecules in the eyes of birds (Ritz *et al.,* 2000). In support of this idea, cryptochromes are expressed in the retina of night-migratory birds (Mouritsen *et al.,* 2004a; Möller *et al.,* 2004), magnetic orientation of night-migratory birds is dependent on the availability of light with wavelengths (e.g. Wiltschko *et al.,* 1993; Wiltschko & Wiltschko, 1999; Munro *et al.,* 1997b; Rappl *et al.,* 2000; Muheim *et al.,* 2002) similar to the absorption spectrum of plant cryptochromes (Lin *et al.,* 1995), and high-frequency oscillating magnetic fields that disturbs the magnetic field effects on radical-pair reactions disrupt magnetic orientation under certain conditions (Ritz *et al.,* 2004; Thalau *et al.,* 2005).

If light-dependent processes in the eyes are involved in magnetoperception, birds should possess a brain area that is specialised in processing visual information at night. In a previous study, we identified such a candidate forebrain area that we named Cluster N, which is specifically activated during the night in night-migratory songbirds (Mouritsen *et al.,* 2005). Blocking visual input inhibited activation in Cluster N. We could not find a well-defined Cluster N in non-migratory birds. Thus, in night-migratory songbirds, Cluster N seems to be a

brain region specialised for enhanced night-time vision, and it could possibly be used to sense geomagnetic field modulations of light-dependent magnetic compass signals and/or to perceive directional information from the stars.

To further elucidate the function of Cluster N and its possible role in processing magnetic information, we measured the expression of neuronal activity dependent immediate early gene (IEG) expression in Cluster N at night following various visual, light, and magnetic field manipulations. Clear differences in the neuronal activation pattern of Cluster N under any of these conditions would indicate involvement of Cluster N in processing light-mediated magnetic compass information, whereas no differences in Cluster N expression under these conditions have to be interpreted with caution (for details see discussion). We also investigated Cluster N in closely related species that show different degrees of migratory behaviour.

Materials and methods

Animals.

We examined two distantly related species of wild-caught night-migratory songbirds, European robins (ER, *Erithacus rubecula*) and garden warblers (GW, *Sylvia borin*), and a closely related non-migratory songbird, Sardinian warbler (SW, *Sylvia melanocephala*). Twenty six European robins were caught around Oldenburg during spring of 2003 and 2004. A total of 43 garden warblers were caught on Helgoland, around Oldenburg, Germany, or in Rybachy, Russia, during autumn 2001-2002 and spring 2003. Five Sardinian warblers were caught in Navarra, Spain in August 2004. All birds were housed indoors, under a simulated local photoperiod. They were kept at least three days in captivity in Oldenburg before testing. Behavioural tests on European robins were performed during the spring migratory season from April 15 to May 25, 2003 and April 14 to 29, 2004. Test on 35 garden warblers were performed during the autumn migratory season from August 22 to October 18, 2002 and 2003. Three garden warblers were tested during the non-migratory season between July 17 and August 3, 2003. The test under monochromatic red light on five garden warblers was carried out during the non-migratory season between December 13 and 22, 2004. The Sardinian warblers were tested during the autumn migratory season between September 11 and October 8, 2004. Some of these animals were also included in the previous experiments (Mouritsen *et al*., 2005). All animal procedures were approved by the Animal Care and Use Committees of the Bezirksregierung Weser-Ems (Oldenburg, Germany).

Behavioural setup.

We used the behavioural set up described in Mouritsen *et al.* (2004a, 2004b, 2005). It consists of a cylindrical, transparent Plexiglas-cage (height 40 cm, diameter 40 cm) with a circular perch (diameter 20 cm) placed 8.5 cm above the ground in the centre of the cage. The cage was placed inside a 2x2x2 m Helmholtz-coil system (Mouritsen 1998), controlled by high-precision constant current power-supplies (Kepco BOP 50-2M). This allowed for controlled manipulation of the magnetic field in any direction. Four small light bulbs placed on the floor provided uniform dim illumination of ~1 mW m^2 intensity (equivalent to 0.04 lux, simulating a moon-lit night). Two infrared-sensitive cameras, providing top and side views of the cage, were connected to a split-screen surveillance monitor to allow for real-time observation and recording to video (25 frames/sec) and PC (5 frames/sec) during day and night. Migratory birds tested in this setup performed stereotyped migratory restlessness behaviour or sat still and awake on the perch. Experiments took place in a wooden house providing access to an undisturbed natural magnetic field (67° ± 1° inclination, 47700 nT ± 600 nT total intensity).

Testing procedure

On the day of testing, a thin stripe of IR-reflective tape (Retroreflective tape, 3M) was glued to the top of the bird's head. The IR tape allowed us to track and analyse the bird's orientation on digital videos using a custom-written MATLAB program. This program computes the position of the bird's head by screening each single frame for the white spot stemming from the reflective tape and relating its position to the centre of the cage. The birds were placed into the cylindrical cage between 12:30h and 13:30h; food was removed 90 minutes before onset of darkness (onset of darkness during the experimental period was between 20:00h and 20:30h). For the day-time control group, the birds were placed into the cage during day-time without food and observation started after approximately 1 hour of habituation to the cage. This habituation period was necessary to let any handling-induced IEG expression decrease to baseline. After a bird performed a minimum of 45 minutes of the desired constant behaviour, i.e. either showing migratory restlessness (or regular movements during the day) or sitting still but awake, the animal was sacrificed by decapitation and the brain was rapidly dissected. The two hemispheres were separated along the mid-sagittal plane, embedded in TissueTek O.C.T. (Sakura Finetek), and quick-frozen to -80°C in a dry ice/ethanol bath.

Lateralisation experiment

In birds, fibers of the optic nerve are completely crossed and interhemispheric commissures are comparatively small (Weidner *et al*., 1985). Thus, visual input from the left eye is

predominantly processed by the right hemisphere and vice versa. Testing birds with monocular light tight eye caps therefore allows the analyses of the extent of visual input perceived in the brain via each eye separately: Monocular eye covers have been suggested to have strong effects on magnetic orientation in European robins (Wiltschko *et al*., 2002). Therefore, we fitted European robins with light tight eye caps (n=8 left eye covered; n=7 right eye covered), built of small black plastic–velour cylinders (diameter=10mm) enlarged with Leucoplast (Beiersdorf AG, Hamburg, Germany) and light-tight black tape. The eye caps were glued to the bird's head so that they covered the eyes completely without any direct contact to the eyes themselves. Controls for lateralisation had either no eyes covered (n=6) or both eyes covered (n=5). The birds wearing unilateral eye caps were given one day to adapt to wearing the eye cap (the eye cover was fitted at noon the day before the experiment) before they were tested in the experiment. For birds wearing bilateral eye caps, these were fixed onto the bird's head three to five hours before onset of darkness. In order to avoid gene expression due to motor behaviour (Feenders *et al,.* personal communication) that might mask small differences in expression level due to magnetic sensing, we only included birds that had been sitting still but awake for a minimum of 45 minutes. Birds of all four groups were tested in a changing magnetic field. This allowed for direct comparison with the garden warblers (see below).

Magnetic field experiment

It is known that redstarts (*Phoenicurus phoenicurus*) cannot orient in a true-zero magnetic field (Mouritsen 1998). Therefore, we placed garden warblers in the cylindrical cage and exposed them to one of three magnetic field conditions (exposure started one hour after onset of darkness, and the birds were exposed to the magnetic field condition for a minimum of 45 minutes): i) natural magnetic field (NMF) during night-time (n=5); ii) changing magnetic field (CMF) during night time with magnetic north switching 120° (clockwise then back) every 5 minutes (n=13); iii) completely compensated zero magnetic field (ZMF) during night-time to eliminate all directional information from the geomagnetic field (n=10) (Mouritsen *et al.,* 2004b). As a control group we tested garden warblers during the day-time in a NMF (NMFday; n=10).

Monochromatic red light experiment

Exposure to monochromatic red light is known to disrupt magnetic orientation (Wiltschko *et al.,* 1993) at least for some time (Wiltschko *et al.,* 2004a). Therefore, we replaced the white light bulbs used to generate the dim light with an array of diodes emitting light in the red range between 600-700nm with a clear peak at 650nm. In previous studies the peak wavelength that

temporarily disrupts magnetic orientation behaviour ranged from 617nm to 635nm (Wiltschko *et al.,* 1993; Rappl *et al.,* 2000; Muheim *et al.,* 2002; Wiltschko *et al.,* 2004a, b). We then tested four garden warblers 45 minutes under this red light (intensity 1.02 mW m^2) in a NMF and compared their Cluster N activation with simultaneously hybridised brain sections of six garden warblers tested in a NMF under dim white light and ten garden warblers tested in a NMF during the day from animals obtained in the previous study (Mouritsen *et al*., 2005). We added one additional individual under the standard dim white light condition just to help normalise any potential differences between experiments.

Sardinian warblers

Three Sardinian warblers were tested under a CMF during night-time in dim white light, and two Sardinian warblers were tested in a NMF during day-time in full room light. We chose CMF in order to allow for direct comparison of the results with previous data collected from garden warblers.

Gene expression analysis.

We used behavioural molecular mapping to visualise neuronal activation in Cluster N and the rest of the brain. Behavioural molecular mapping relies on the expression of IEGs to identify functional areas in the brain showing an increase in neural activation during specific behaviour and processing tasks (Jarvis & Nottebohm, 1997). We used in-situ hybridisations to visualise expression patterns of the IEG called ZENK (for methodological details see Wada *et al.,* 2004 and Mouritsen *et al*., 2005) in 12 µm frozen sections. For the magnetic field condition experiments, frozen sections were cut throughout the whole brain of one individual per group: the left hemisphere in the sagittal plane and the right hemisphere in the frontal plane. As no magnetic-condition-dependent differences were observed between the two hemispheres, we continued to cut the left hemisphere only in the remaining birds. We chose the sagittal plane as it provided the best overview of the Cluster N expression pattern. The sections were fixed in 4% paraformaldehyde and processed by *in-situ* hybridisation with antisense S^{35}-UTP riboprobes made from a zebra finch ZENK cDNA. Expression of ZENK mRNA is driven by neuronal activation and occurs throughout the brain at high levels except in primary thalamic recipient neurons of the forebrain (L2, entopallium, and nucleus basorostralis), the globus pallidus, and parts of the thalamus (Mello & Clayton, 1995; Jarvis, 2004). Accumulated mRNA can be detected in neurons approximately 10-60 minutes after activation occurred, with a peak after circa 30-45 minutes (Jarvis & Nottebohm, 1997). The hybridised sections were first exposed to x-ray film (Biomax, Kodak) and then dipped into autoradiographic emulsion

(NTB2, Kodak) diluted 1:1 with water, incubated for 4-6 weeks at 4°C, developed using Kodak developer (D19) and fixer, Nissl stained with cresyl violet acetate (Sigma), and coverslipped with permount glue (Fisher). For quantification, the x-ray films of brain images were digitally scanned using a dissecting microscope and either Diagnostic Instruments SPOT III or Leica DFC320 camera. Using the ADOBE PHOTOSHOP (Adobe Systems Inc.) software histogram tools, brain regions of interest were encircled and the mean pixel density was measured on a 256-level grey scale.

Quantification of ZENK expression in Cluster N was done as previously described (Mouritsen *et al.,* 2005): the relative pixel density of the Cluster N area (comprising posterior-dorsal parts of the hyperpallium apicale [HA], intercalated hyperpallium apicale [IHA], dorsal mesopallium [MD], the dorsal nucleus of the hyperpallium [DNH] and DNH-shell) was subtracted by the values of the anterior-ventral part of HA, IHA and MD (see Figure 3A, B). In the lateralisation experiments, we directly quantified the difference in Cluster ZENK activation (pixel density) between hemispheres of the same bird on the same slide (unless otherwise noted).

Results

Lateralisation

To further investigate the role of vision in Cluster N activation, we performed monocular and binocular eye cover experiments with European robins. Prior to covering the eyes, as a group, the European robins showed correct magnetic orientation directed towards northeast consistent with the birds' natural spring migratory direction (Fig. 1A; $\alpha=38°$, $r=0.64$, $p<0.001$). No celestial cues were available to the birds so they had to rely on magnetic field information, only. Birds with monocular eye covers showed less activation of Cluster N in the brain hemisphere contralateral (opposite) to the covered eye (Fig. 1B, D; one-way ANOVA, followed by Holm-Sidak multicomparison test: $p<0.001$). However, ZENK activation in the contralateral Cluster N (with respect to the eye cover) was still significantlty higher than the levels seen in the corresponding hemisphere of birds that had both their eyes covered (Fig. 1B, D; t-test comparing the contralateral expression [with respect to the eye cover] in birds with one eye covered to the expression in the equivalent hemisphere in birds with both eyes covered, $p=0.005$). This significant activation of Cluster N in the hemisphere that receives its main input from the occluded contralateral eye is presumably due to ipsilateral (same side) projections from the open eye.

The extent of ipsilateral Cluster N activation is of the same order of magnitude for both unilateral eye cover conditions (t-test, p=0.94; comparing Cluster N activation of the left hemisphere for birds with the right eye covered and the right hemisphere of birds with the left eye covered). In contrast, the contralateral activation of Cluster N between the hemispheres of birds with one eye covered was significantly higher when the right eye was covered than when the left eye was covered (Fig. 1C; t-test, p=0.021, comparing left minus right hemisphere for left eye covered birds with right minus left hemisphere for right eye covered birds). This asymmetry was also present in birds with both eyes open (Fig. 1B, D), where ZENK activation in the right Cluster N of European robins is significantly higher than in the left Cluster N (see Fig. 1D; t-test, p=0.007, within-bird comparisons). When both eyes are covered, in addition to the ZENK decrease in both hemispheres, the hemispheric asymmetry disappears (Fig. 1B, C; t-test, p = 0.989). These results indicate that, in European Robins, Cluster N is a lateralised night-vision brain area that is right dominant.

Magnetic conditions

To investigate a possible role of Cluster N in magnetic field processing, we placed garden warblers in the cylindrical cage and tested them under different magnetic field conditions. We chose garden warblers, as this is the species in which Cluster N was initially characterised (Mouritsen *et al.,* 2005). Prior to the ZENK mapping experiments, as previously described (Mouritsen *et al.,* 2004b, 2005), our garden warblers oriented towards southwest in autumn under NMF whereas they did not show significant directional orientation under ZMF (Fig 2A,B). Thus, our birds did use the magnetic field for orientation. However, the expression level of ZENK in Cluster N did not differ significantly between birds that were exposed to NMF, ZMF, or CMF (Fig. 2C, one-way ANOVA, followed by Holm-Sidak multicomparison test: NMF *vs.* ZMF, p=0.085; NMF *vs*. CMF p=0.145; CMF *vs.* ZMF, p=0.660). All three groups were significantly different from the day-time NMF group (Fig. 2C, one-way ANOVA, followed by Holm-Sidak multicomparison test: p<0.001).

Monochromatic red light

To determine if the wavelength of dim-light is important for Cluster N neuronal activation, we tested garden warblers exposed to red light. Red light exposure with a peak wavelength from 617nm to 635nm has previously been shown to temporarily disrupt magnetic orientation behaviour in garden warblers (Wiltschko *et al.,* 1993; Rappl *et al.,* 2000; Muheim *et al.,* 2002; Wiltschko *et al.,* 2004b; but see Wiltschko *et al.,* 2004a for a weakness of this result). ZENK expression in Cluster N in garden warblers exposed to dim red light did not differ significantly

from the expression seen under dim white light; in red light, ZENK expression was still significantly higher than the expression level during the day (Fig. 3, one-way ANOVA followed by Holm-Sidak multicomparison test; red light *vs.* white light: p=0.342; red light *vs.* day: p<0.001). Since we conducted these experiments during the winter, when garden warblers are not migrating, the results provided further evidence that Cluster N is activated to a similar degree throughout the year (Mouritsen *et al.,* 2005). The present data complete the annual cycle to show that Cluster N activation is neither affected by season nor by migratory behaviour itself.

Sardinian warblers

We found high activation of Cluster N in fairly sedentary Sardinian warblers tested in CMF under dim white light at night (Fig. 4A). The ZENK expression pattern in Cluster N was very similar to that seen in garden warblers and European robins. Cluster N ZENK expression levels in Sardinian warblers during night-time was significantly higher than during day-time (Fig, 4C, t-test, p=0.005, t=-7.677). However, in Sardinian warblers during day-time, the shell around the DNH showed high ZENK expression especially in one of the two birds tested (not shown). This was never observed in the garden warblers. The reason for this variation in expression patterns remains unclear.

Discussion

Recently, we described Cluster N as a brain area that is specifically activated during night time in night-migratory songbirds (Mouritsen *et al.,* 2005). We suggested that this brain area could be involved in special night vision and possibly in information processing of magnetic field and/or star compass orientation. In the present study, our findings do not add closure as to whether Cluster N is involved in processing of magnetic information, but they do strengthen the conclusion that Cluster N is a night-time vision brain area specialised in primarily migratory clades of birds.

The eye cover experiments revealed a marked dominance of the right hemisphere in light-induced activation of Cluster N. Since the extent of the ipsilateral Cluster N activation does not show the asymmetry we observed for total Cluster N activation, the dominance in Cluster N activation of the right hemisphere is presumably due to contralateral rather than ipsilateral projections. This observation adds to the mounting evidence that shows that there are asymmetries in the organisation of visual pathways in birds (e.g. Bischof & Watanabe, 1997;

for a review see Rogers, 1996; Güntürkün, 2002, 2006). Several studies have demonstrated a preferential use of the right eye and left hemisphere in food discrimination of chicks, pigeons, and zebra finches (Alonso, 1998; Güntürkün & Kesch, 1987; Mench & Andrew, 1986), and the left eye and right hemisphere in social recognition (Vallortigara 1998). Based upon these findings it has been proposed that, in birds, the left hemisphere is involved in sustaining attention on an object, while the right hemisphere is used to analyse details and control rapid responses (George *et al*., 2006). If a similar property exists for Cluster N, then intuitively processing of magnetic field and/or starry sky may require attention to details, a right visual brain function.

Our findings suggesting dominance for Cluster N of the left-eye/right-hemisphere seem to be in conflict with behavioural experiments, which report a dominance of the right-eye/left-hemisphere in magnetic compass orientation behaviour (Wiltschko *et al.,* 2002). One possibility is that Cluster N is not involved in processing magnetic compass information. However one or more forebrain areas should be processing magnetic compass information in birds performing magnetic compass orientation and should be detectable as an active region by means of behavioural molecular mapping. Despite a careful analysis of the entire brain, we found no forebrain region other than Cluster N showing dominant and consistent ZENK expression during night time magnetic compass orientation (Mouritsen *et al.,* 2005). Thus, the discrepancy between the behavioural results suggesting right-eye/left-hemisphere exclusivity in magnetic compass orientation and the activation patterns observed in the forebrain during magnetic compass orientation are difficult to explain.

The data on garden warblers tested under various magnetic field conditions, including a completely compensated ZMF, revealed consistently high immediate early gene expression in Cluster N. The ZMF group did show the lowest ZENK expression levels, but differences between the magnetic field groups at night were non-significant. If Cluster N is integrating magnetic field information, one could assume this area to show a distinctly different activity pattern because a true-zero magnetic field scenario does not provide any magnetic information at all. But if we assume that the underlying receptive mechanism is light-based, then the light itself is the primary signal on which the magnetic field imposes small modulations. Consequently, then whenever light is available – as it was in our magnetic field study – radical-pairs should be formed as a result of photo-excitation. In a NMF, there is expected to be small modulations (probably in the order of a few percent) of the singlet-triplet proportions in different parts of the retina (Ritz *et al*., 2000). In a ZMF, the radical-pairs are expected to occur in a default proportion of singlet and triplet states throughout the eye and independently of the orientation of the radical-pair-forming molecules. In both NMF and ZMF, the light induced

signals will have to be sent to the brain for further processing (e.g. to compare the input from different parts of the retina) irrespective of the magnetic field condition. Therefore, if the putative magnetic modulations are generally small and/or modulated in opposite directions, behavioural molecular mapping may not be sensitive enough to detect magnetic modulations of neuronal signals in Cluster N that could occur under different magnetic field conditions. The IEG expression indicates the average expression level in all neurons within a certain brain area over a time span of ca. 45-60 minutes. Thus, the lack of differences in Cluster N ZENK activation in the manipulated magnetic field conditions does not rule out Cluster N for a role in processing of magnetic compass information.

Birds exposed to monochromatic red light showed prominent ZENK expression in Cluster N at night. The activation in Cluster N under red light seemed to be slightly lower than under white light, but this difference was not significant. Initially, red light was shown to temporarily disrupt magnetic orientation under certain conditions (Wiltschko *et al.,* 1993, 1998, 2001; Wiltschko & Wiltschko, 1999, 2001b). However, recently Wiltschko *et al.* (2004a) subsequently showed that when European robins are pre-exposed to the red light for 1h, the birds orient well. Thus, possible explanations for the robust Cluster N activation we found in our birds tested under red light are: (A) the primary sensor is sensitive to red light, but the brain needs time to interpret the unusual signals correctly, or (B) the 45 minutes of pre-exposure to red light have been long enough to modify the sensitivity of the primary receptor to respond to red light, or (C) a signal originating from an alternative receptor system (for discussion, see Wiltschko *et al.,* 2004a) leads to Cluster N activation. The latter interpretation is in line with recent findings supporting the suggestion that at least two different receptor-types exist in the retina of birds (Wiltschko *et al.,* 2004a, b). It could be that a second receptor-type gets activated under our red light condition and sends a signal to the brain, which is reflected as Cluster N activation.

Our finding that the neuronal activation of Cluster N is seen both during migratory and non-migratory seasons suggest that the specialised night vision processing can occur at all times of the year. It is commonly observed in other seasonally relevant sensory systems that sensory input is processed year around, even during seasons when the information is not of behavioural relevance. For example in canaries, which are seasonal breeders, ZENK is equally strongly activated in auditory forebrain areas year round (Jarvis & Nottebohm, 1997), even though for this and other species, song processing is more relevant during the breeding season and leads to important behavioural responses at that time (Nottebohm *et al.,* 1987).

The experiment with Sardinian warblers revealed significant neuronal activation in Cluster N at night but not during the day, thus providing a similar pattern of Cluster N

activation to that observed in garden warblers. Sardinian warblers are fairly sedentary in the Mediterranean and are frequently used as a non-migratory *Sylvia*-warbler in comparative studies (e.g. Healy *et al.,* 1996; Mettke-Hofmann & Gwinner, 2003). Nevertheless, it has been observed that individuals of this species undertake directed movements in autumn and spring (Cramp, 1998), and some of our birds actually performed some migratory restlessness at night. Although there is no evidence in the literature about how Sardinian warblers may orient, it is likely that this species is capable of orienting e.g. according to the magnetic field. In fact, non-migratory birds can also use a magnetic compass (homing pigeons, *Columba livia*: Keeton, 1971; Wiltschko & Wiltschko, 2001a; chick, *Gallus domesticus*: Freire *et al.,* 2005a, b), but it is not yet clear if the mechanism is the same in night migrants and non-migrants. Further evidence that at least a partial migratory behaviour is likely to exist in Sardinian warblers is provided by the studies of Pulido, Berthold, and colleagues (Pulido *et al.,* 1996). They have shown that within natural populations of blackcaps (*Sylvia atricapilla*) and probably many other species there exist individuals showing more migratory behaviour, less migratory behaviour, or no migratory behaviour at all, and that many populations can evolve into a migratory or non-migratory state within a few generations. Therefore, it is likely that all species in a predominantly night-migratory clade of birds, such as the *Sylvia* warblers, posses the ability to orient according to a magnetic compass because sedentary/migratory behaviour has probably evolved and re-evolved repeatedly over the last 10,000 years (review: Berthold 1999; Piersma *et al.,* 2005). Consequently, predominantly migratory clades are likely to possess the ability to orient at night using the magnetic field and they would therefore be expected to have a functional Cluster N if it is indeed used for integrating light-mediated magnetic compass information.

In conclusion, our results suggest a marked dominance of the right hemisphere in processing the visual component integrated in Cluster N. The signal sensed and the information asymmetrically processed in Cluster N is light mediated. If the vision-mediated information processed and integrated in Cluster N were of magnetic nature, this would be in line with theoretical (Ritz *et al.,* 2000), molecular (Mouritsen *et al.,* 2004a), and behavioural (e.g. Ritz *et al.,* 2004; Mouritsen *et al.,* 2004b) evidence suggesting that the Earth's magnetic field modulates the light sensitivity of specialised receptor molecules differently in various parts of the retina. Tests under various magnetic field conditions and under red light could not be used to clarify whether Cluster N is an integrative area for magnetic compass orientation, or whether it is 'just' a night vision area specialised in night-migratory species, probably including their close relatives. A specialised night vision area may be necessary to extract directional information from the starry sky, or it may trigger the bird's alertness to initiate night-migratory

behaviour. Electrophysiological recordings and studies with lesions of Cluster N will be crucial to elucidate the ultimate function of Cluster N.

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Abbreviations

A	arcopallium
CMF	changing magnetic field
DNH	dorsal nucleus of the hyperpallium
E	entopallium
ER	European robin, *Erithacus rubecula*
GW	garden warbler, *Sylvia borin*
H	hyperpallium
HA	hyperpallium apicale
HF	hippocampal formation
ICo	inferior colliculus
IEG	immediate early gene
IHA	intercalated hyperpallium apicale
IR	infrared+
L2	field L2
M	mesopallium
MD	dorsal mesopallium
MV	ventral mesopallium
N	nidopallium
NMF	natural magnetic field
OT	optic tectum
P	pallidum
St	striatum
SW	Sardinian warbler, *Sylvia melanocephala*
T	Tesla
V	ventricle
W	visual Wulst.
ZENK	bird acronym; gene is known in other species as *zif-268, egr-1, NGF-IA* and *krox-2*
ZMF	zero magnetic field

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Figure Legends

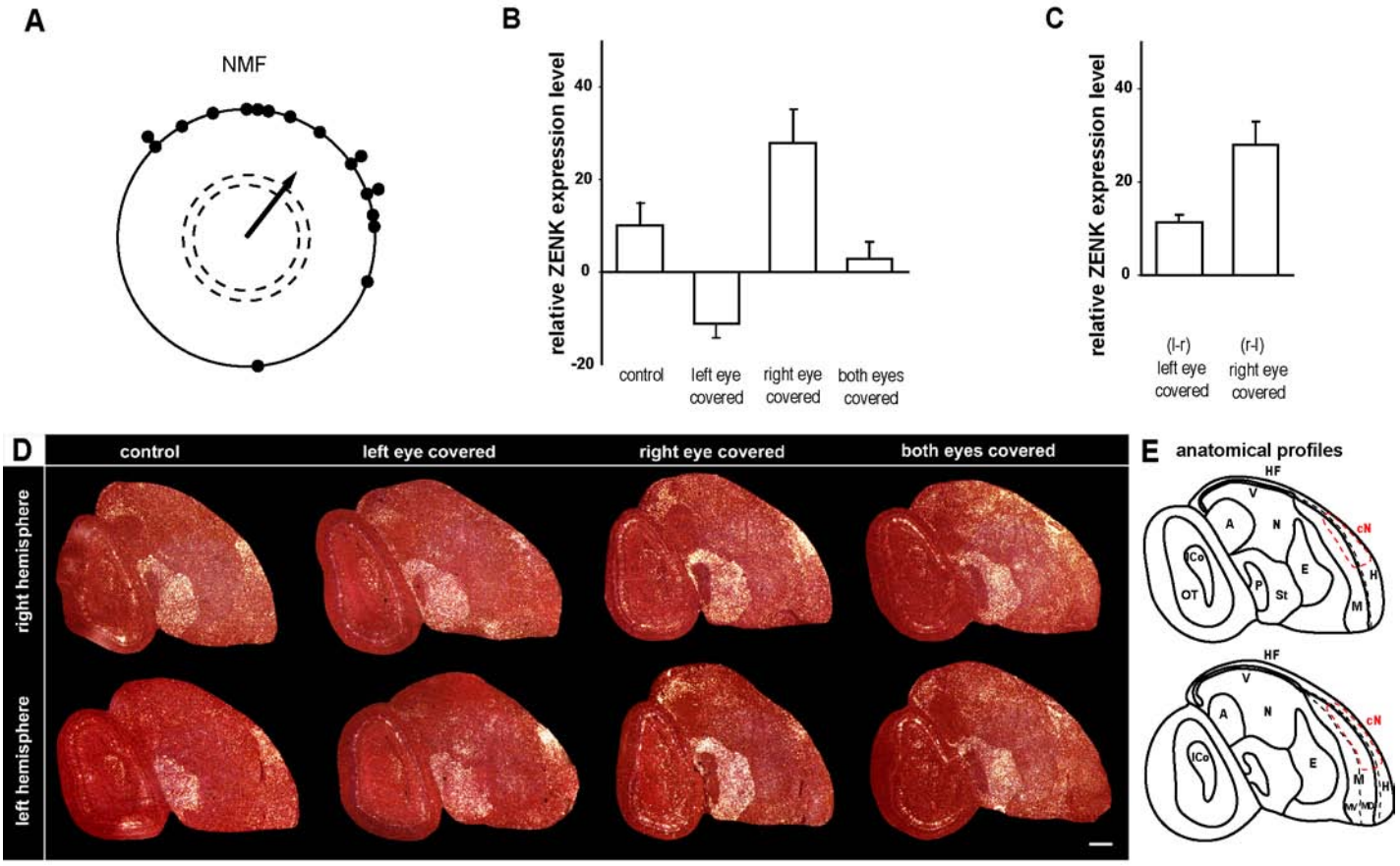
Figure 1: ZENK expression in European robin brains under different eye cover conditions. *A* Orientation of European robins during spring migration in a natural magnetic field. Each dot represents the mean orientation of one individual bird. The arrow indicates the mean orientation of the group. The length of the arrow represents the length (r-value) of the group mean vector. The dashed circles indicate the radius of the group mean vector needed for significance ($p<0.05$ and $p<0.01$) according to the Rayleigh Test of uniformity. *B* Relative ZENK expression differences between the right and left hemisphere for control birds with both eyes open (0), birds wearing either a left (L) or right eye cover (R), and birds with both eyes covered (LR). One way ANOVA comparison between all groups revealed highly significant differences for all group comparisons ($p<0.001$), except for the comparison between the two control groups: birds with both eyes open and birds with both eyes covered. *C* Direct comparison between hemispheres for birds with unilateral eye covers suggesting a marked dominance of the right hemisphere Cluster N over the left hemisphere Cluster N ($p=0.021$). *D* Dark field image of ZENK expression in saggital sections showing Cluster N expression under different eye cover conditions. Dorsal is up, rostral is right. *E* Anatomical profile of the right image (condition: both eyes covered) shown in *D*. Error bars: standard error. Scale bar: 1 mm. A: arcopallium; E: entopallium; HF: hippocampal formation; H: hyperpallium; ICo: inferior colliculus; IHA: intercalated hyperpallium apicale; M: mesopallium including ventral (MV) and dorsal (MD) part; N: nidopallium; OT: optic tectum; P: pallidum; St: striatum; v: ventricle; W: visual Wulst.

Figure 2: ZENK expression in garden warbler brains under different magnetic field conditions. *A* & *B*: Orientation of garden warblers during autumn migration in a natural magnetic field (*A*) and a compensated true-zero magnetic field (*B*), symbols as in Figure 1. *A* & *B* reproduced with permission from *Current Biology,* since the results reported here originate from the same individual birds as used in Mouritsen *et al.,* 2004b. *C*: Relative ZENK expression levels in Cluster N in birds exposed to natural (NMF), changing (CMF) or zero magnetic field (ZMF) compared with birds collected during day-time (day, NMF). Error bars: standard error.

Figure 3: ZENK expression in garden warbler brains under white versus red light. *A*: Darkfield image of Cluster N ZENK expression under dim white light at night (*left*) and after 45 min exposure to monochromatic red light (*right*). Dorsal is up, rostral is right. *B*: Anatomical profile of the right image shown in *A*. *C*: Quantification of ZENK expression level in Cluster

N, comparing exposure to standard white light with red light. Error bars: standard error. Scale bar: 1 mm. For abbreviations see Figure 1.

Figure 4: ZENK expression in Sardinian warblers. *A*: Darkfield image of ZENK expression in a sagittal section showing Cluster N. Dorsal is up, rostral is right. *B*: Anatomical profile of the image shown in *A*. The red dashed line marks the boundary of Cluster N. *C*: Quantification of ZENK expression level in Cluster N, comparing day-time and night-time group. Error bars: standard error. Scale bar: 1 mm. For abbreviations see figure 1.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NMF
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control
left eye covered
right eye covered
both eyes covered
C
relative ZENK expression level
40
20
0
(l-r) left eye covered
(r-l) right eye covered
D
control
left eye covered
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both eyes covered
right hemisphere
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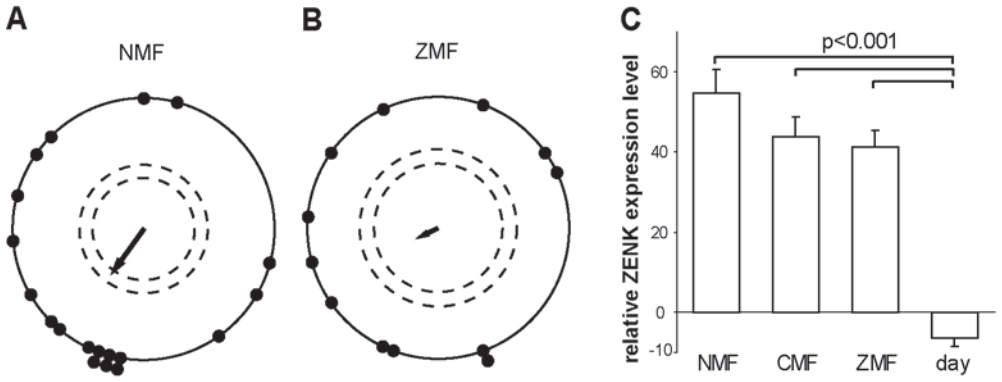
Figure 1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NMF
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relative ZENK expression level
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-10
NMF
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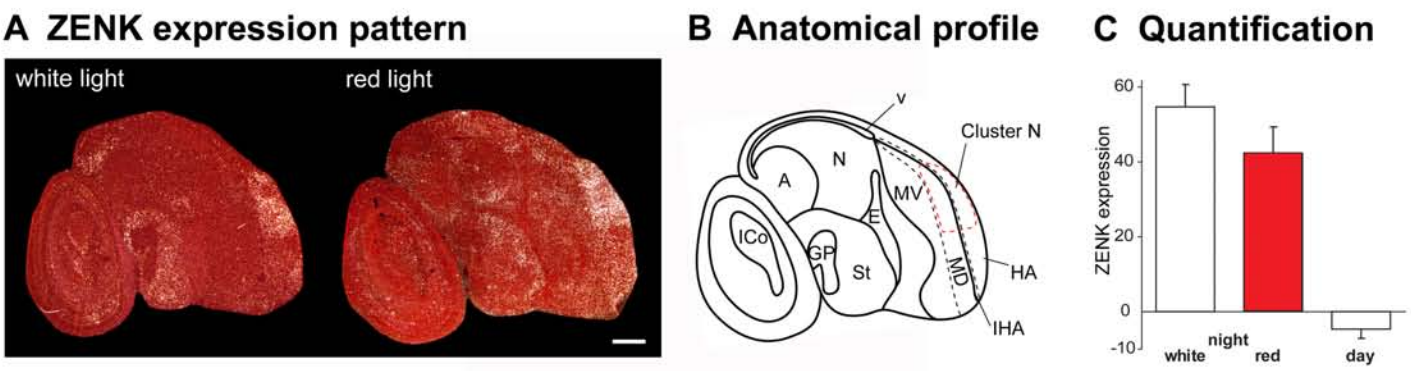
day

Figure 2


A ZENK expression pattern
white light
red light
B Anatomical profile
v
Cluster N
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C Quantification
ZENK expression
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night
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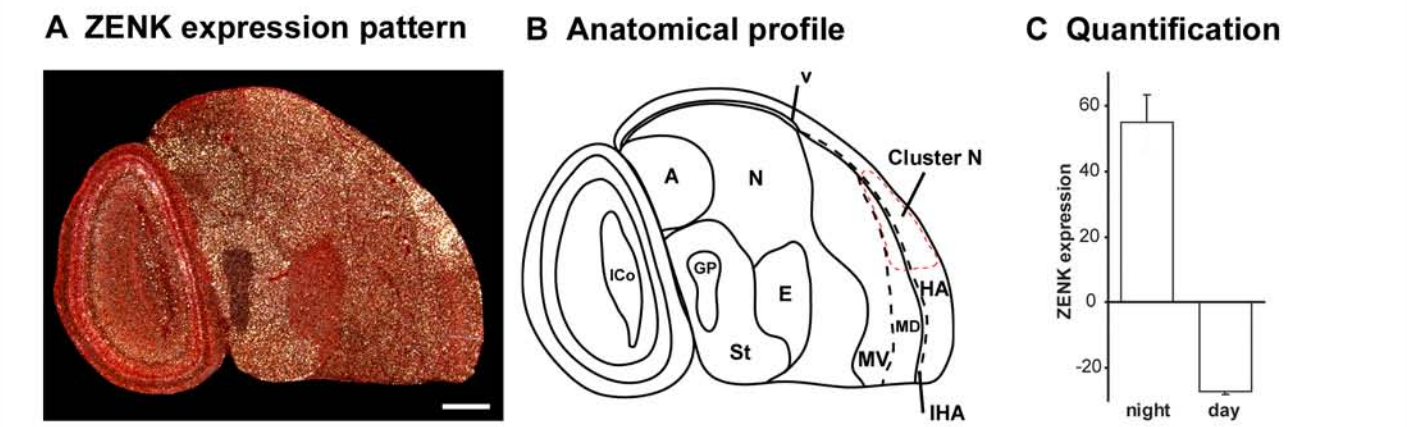
Figure 3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B Anatomical profile
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ZENK expression
night
day

Figure 4

Paper VI

Feenders, G., Liedvogel, M., Wada, K., Mouritsen, H. & Jarvis, E.D.:

Movement-driven gene expression patterns implicate origin of brain areas for vocal learning.

under review, 2006.

Movement-driven gene expression patterns implicates origin of brain areas for vocal learning

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Running Title: Movement in the brain

Brain structures and pathways that control vocal learning in birds are strikingly similar among distantly related groups, even though they are not found in their closely related vocal non-learning relatives. This led to the hypothesis that vocal learning brain pathways evolved independently in the distantly related groups but under pre-existing constraints. Here, we show what this constraint may have been. We studied movement-driven gene expression in migratory songbirds displaying migratory restlessness behavior and in non-migratory songbirds displaying general movements. We discovered that in songbirds, migratory or not, vocal learning brain nuclei active during vocalizing are embedded in or adjacent to brain areas active during head, wing, and body movements. We also found high activation in the anterior medial hyperpallium apicale and subdivisions of the cerebellar cortex, consistent with known somatosensory pathway inputs. These movement activated brain areas were also present in female songbirds that do not have vocal nuclei nor learn vocalizations. These findings present the first global mapping of avian brain motor areas and suggest that vocal learning brain systems evolved out of a pre-existing motor pathway that controls movement.

Key words: vocal pathway, language, migration, migratory restlessness, flight, ZENK

Introduction

Vocal learning, the behavioral substrate for human language, is a rare trait to date found in three distantly related groups of birds: songbirds, hummingbirds, and parrots (**Fig. 1A**). In these groups, vocalizing-driven immediately early gene (IEG) expression has revealed that each, remarkably, possess exactly seven cerebral vocal nuclei active in the production of learned vocalizations (**Fig. 1B**;Jarvis et al., 1998; Jarvis and Mello, 2000; Jarvis et al., 2000). Three of the nuclei form a column within the anterior vocal pathway (**AVP; Fig. 1B, red**), a pathway that in songbirds is necessary for vocal learning (Scharff and Nottebohm, 1991). The other four nuclei form a posterior vocal pathway (**PVP; Fig. 1B, yellow**), a pathway that in songbirds is necessary for the production of the learned vocalizations (Nottebohm et al., 1976; Simpson and Vicario, 1990). None of these seven cerebral vocal nuclei have been found in vocal non-learners (Kroodsma and Konishi, 1991; Haesler et al., 2004; Wada et al., 2004). Yet, both vocal learners and non-learners have brainstem vocal nuclei DM and nXIIts (**Fig. 1B**) that are responsible for production of innate vocalizations (Wild, 1997a; Jarvis et al., 2000). Therefore, the implications are remarkable: either vocal learning evolved multiple independent times and each time, it did with seven similar cerebral brain structures; or vocal learning was present in a common avian ancestor ~65 million years ago and then was lost multiple independent times (Jarvis et al., 2000).

In either case, the vocal learning brain pathways in birds share notable similarities with motor brain pathways in mammals (Perkel and Farries, 2000; Jarvis, 2004a; Jarvis, 2004b; Doupe et al., 2005). The AVP (**Fig. 1B, white arrows**) is similar in connectivity and function to mammalian pre-motor cortical-basal-ganglia-thalamic-cortical loops. The PVP (**Fig. 1B, black arrows**) is similar in connectivity and function to the mammalian primary motor pathway where the motor cortex sends projections to midbrain, hindbrain,

and spinal cord non-vocal motor neurons. The PVP sends a major projection from the arcopallium (RA in songbirds) to midbrain (DM) and hindbrain (nXIIts) vocal neurons, the latter controlling muscles of the avian vocal organ - the syrinx. For both pathways, neuron populations adjacent to vocal nuclei, as well as in comparable locations in vocal non-learning birds have been noted to form similar connections as the vocal nuclei (Margoliash et al., 1994; Iyengar et al., 1999; Farries, 2001; Diekamp and Gunturkun, 2003; Jarvis, 2004b; Doupe et al., 2005; Farries et al., 2005). However, the functions of most of these adjacent regions are not known. The above findings taken together have been used to suggest there may have been a pre-existing constraint on the evolution of vocal learning to develop out of a conserved network, as one possibility among several to explain evolution of brain pathways for vocal learning (Farries, 2001; Jarvis, 2004b). This study shows that the vocal learning brain nuclei of songbirds are embedded within or adjacent to brain areas that are specifically active in the generation of movement, and thus vocal learning pathways seem to have evolved out of a pre-existing motor system. This is a logical way to evolve vocal learning, because the production of new vocalizations requires precise control of syringial muscles.

Materials and Methods

Behavior apparatus. General behavior of birds includes a variety of motor activities that are difficult to quantify and not always consistently performed. Thus, in order to use behavioral molecular mapping to identify motor activated brain areas, as done for singing (Jarvis and Nottebohm, 1997), it is necessary to find a behavioral paradigm that allows consistent and repetitive motor behavior by the animal suitable for quantification. One such highly stereotyped behavior is migratory restlessness (MR), performed by migratory

songbirds at night. During their migratory season, caged birds perform wing whirring and other consistent movements in a preferred direction (Kramer, 1949; Emlen and Emlen, 1966) corresponding to the migratory orientation of their free-flying conspecifics (Mouritsen, 1998; Mouritsen and Larsen, 1998). To reliably record and quantify MR and non-migratory movement behavior in songbirds and relate this behavior to activity-dependent gene regulation, we designed a behavior apparatus consisting of a cylindrical, transparent Plexiglas-cage (height 40 cm, diameter 40 cm) with a circular perch (diameter 20 cm) placed 8.5 cm above the ground in the center of the cage (**Fig. 2**; Mouritsen et al., 2004a; Mouritsen et al., 2005). This apparatus allowed us to carefully observe the bird's behavior in real time. We found that migratory songbirds during dim light either sit in this orientation cage for extended periods of time or perform MR behavior more consistently and stereotypically than they do in the Emlen funnels (Emlen and Emlen, 1966) normally used for orientation experiments. We also found that the migratory birds use head scans apparently to detect the direction of the Earth's magnetic field (Mouritsen et al., 2004a; Mouritsen et al., 2004b). Zebra finches, a non-migratory songbird species, would mostly run around in the cage in day light conditions, or just sit in dim light conditions.

Animal groups. A total of 38 garden warblers (*Sylvia borin*; 29 males and 9 females; migratory songbirds) and 10 zebra finches (*Taeniopygia guttata*, 9 males and 1 female, non-migratory songbirds) were used for this study. The garden warblers were caught on Helgoland and around Oldenburg, Germany, in May-September, 2002/2003, and acclimated to captivity for a minimum of 5 days. Zebra finches were purchased from a local breeder. Behavioral tests were performed during the autumn migratory season for warblers, between August 22 and October 18, 2002 and 2003, and during the non-migratory season between July 17 and August 3, 2003. On the day of testing, a thin stripe

of IR-reflective tape (Retroreflective tape, 3M) was glued to the top of the bird's head. The IR tape was used to track and record the bird's movements with an infrared-sensitive video camera in dim light. Tracking movement behavior in dim light versus day light allowed us to separate out movement-induced from day light-induced gene expression. For the dim light conditions, the birds were placed into the cylindrical cage before 13:30h and food was removed 90 minutes before onset of darkness (simulated local photoperiod). Some birds were exposed to an artificially changed magnetic field, but these manipulations did not affect the IEG expression in motor activated areas, and the results of these conditions will therefore be described elsewhere.

To separate sensory (visual) from motor components of movement behavior we collected birds from four groups: 1) animals that sat relatively still in the cage during the day time, showing a minimum of movement behavior (e.g. less than 5 flights, 100 wing beats, and/or 50 head scans in 1h); 2) animals that remained awake and relatively still during the night in dim light conditions (0.04 lux) for an extended period of time; 3) animals that displayed general motor activity during the day (e.g. several hundred to several thousand defined movement events); and 4) animals that displayed MR motor behavior during the night. In addition, we analyzed two groups of non-migratory zebra finches to control for migration-specific activation: one group displaying regular motor activity during the day and one group sitting still but awake at night. All animal procedures were approved by the Bezirksregierung Weser-Ems and/or the Duke University Institutional Animal Care and Use Committee.

Behavior observation and recording. The behavior of all birds was recorded by the infrared video cameras to videotape (25 frames/sec). In parallel, we carefully observed the birds' behavior in real-time on a split-screen surveillance monitor from 1h before the start

of an experiment and until the experiment was finished. The 1h waiting period allowed the decay of any gene expression induced either by behaviors before the observation period or by the light-dark transition (Jarvis and Nottebohm, 1997). We only collected a bird after it produced repetitive movement behavior or was sitting still but awake while a minimum of other behaviors occurred for at least 45 min (mostly for 1h or more), the peak time of ZENK mRNA expression (Mello and Clayton, 1994; Jarvis and Nottebohm, 1997). To avoid hearing-driven gene expression (Mello et al., 1992; Jarvis and Nottebohm, 1997) due to noise, we made sure that no sounds from the experimenters or from the outside reached the birds during the critical hour. In case of accidental noise (from indoors or outdoors), we waited at least 1h before starting the experiment again. When at least 45 min of the required highly consistent behavior was observed, we sacrificed the bird, rapidly dissected its brain, separated the two hemispheres along the mid-sagittal plane, embedded them in TissueTek O.C.T. (Sakura Finetek, NL), and quick-froze them to -80°C in a dry ice/ethanol bath within 6-12 min after taking the bird out of the cage.

Gene expression analyses. 12µm frozen sections were cut almost throughout most of the left hemisphere, using sagittal sections to maximize the amount of brain tissue per section. Corresponding sections of all birds were fixed in 4% paraformaldehyde and processed by in-situ hybridization with antisense S^{35}-UTP labeled riboprobes of a zebra finch ZENK (acronym for zif268, Egr-1, NGF-1A, and Krox-24) or GluR1 (glutamate receptor 1) cDNA following previously described procedures (Wada et al., 2004). We used GluR1 as an anatomical marker in combination with Nissl staining to identify anatomical boundaries of brain structures. Expression of ZENK mRNA in the brain is driven by neuronal activity - except in primary thalamic recipient neurons of the cerebrum (L2, entopallium, and basorostralis), the globus pallidus, and parts of the thalamus (Mello and Clayton, 1995;

Jarvis, 2004a). ZENK can be detected in neurons about 10 min after onset of neuronal activity with peak expression after 30-45 min (Jarvis and Nottebohm, 1997), and therefore increased cumulative mRNA expression marks brain areas that were active during the last 45-60 min of the birds behavior. The hybridized sections were first exposed to X-ray film (Biomax MR, Kodak) for 1-4 days, then dipped into autoradiographic emulsion (NTB2, Kodak), incubated for 3 to 4 weeks at 4°C, processed with Kodak developer (D-19) and fixer, and Nissl-stained with cresyl-violet acetate solution (Sigma). Sections of one in-situ hybridization experiment were treated identically (exposure time & staining) to allow for direct comparisons. X-ray film brain images were digitally scanned from a dissecting microscope connected to a SPOT III CCD camera with SPOT Advanced imaging software (Diagnostic Instruments, Inc.). For quantification, a person naïve to the experimental conditions used Adobe Photoshop 6.0 to measure the mean pixel intensities in the brain areas of interest on a 256 grey scale. To test for significant differences between individual groups, the SigmaStat software (SPSS, Inc.) package was used to perform ANOVAs followed by a Holm-Sidak multi-comparison, or regression tests. We excluded two garden warblers from the analysis, as the in-situ signal on those sections was very weak and not reliable.

Behavior analysis. We defined and quantified the most prominent types of movements. Some specific movement behaviors of the two species in the cylindrical cage were different:

Number of wing-beats (garden warbler and zebra finch): During the dim light conditions, when garden warblers perform constant MR in our apparatus, they whirred their wings rapidly while perched. During the day, they will flap their wings while perched at a much lower rate and often in preparation to fly off the perch. Zebra finches only

occasionally flapped their wings while perched. We consider wing whirring and flapping all as wing beats. The amount of wing beats during flapping was relatively simple to quantify manually, as the birds performed it at a slow rate. The amount during whirring was not. Thus, we first measured the mean wing beat frequency during constant MR for several birds by watching the video frame-by-frame. This mean frequency (11 wing-beats/sec) was then transferred to the keys of a PC keyboard so that when a key assigned to a Matlab program was pressed continuously, the output signal was identical to the wing-beat frequency. Thereafter, the observer watched all video tapes in real-time and either held the key down for the time the bird performed constant wing-whirring or flying around in the cage or made individual key-strokes for isolated wing flaps while perched. The time and number of each button-press event were analyzed using a custom-written Matlab program.

Number of flights (garden warbler): We reviewed the same videos again and instead of counting the number of wing beats, we counted each time the bird flew off the perch or off the bottom of the cage. Zebra finches were excluded from this quantification as they hardly ever performed flights in the cylindrical cage.

Number of head scans (garden warbler): We reviewed the same videos again and counted head scans. Garden warblers perform head scan behavior, particularly during MR in dim light, apparently to sense the magnetic field (Mouritsen et al., 2004a). The birds typically turn their head to the left or the right, sometimes alternating. We defined one head scan as a head turn of more than 60° to the left or right and approximately back to the longitudinal body axis (sometimes immediately followed by a head scan to the opposite side) within a few seconds while the bird remained at the same position in the cage. We did not observe such consistent head scan behavior in zebra finches.

Running (zebra finches): Zebra finches tended to run around along the perimeter of our cylindrical apparatus. Because this was a persistent behavior, we found it easier to measure by subtracting the time periods the bird was sitting still for 5 sec or more from the 1h total observation interval.

Number of perch jumps (zebra finches): Instead of making flights, zebra finches tended to hop from one spot of the round perch to another. We counted every time the bird hopped across the perch covering more than 45 ° or on/off the perch.

Results

Gene activation as a result of movement behavior

We found that relative to birds that were still, garden warblers that performed non-vocal movement behavior, during the day or night, had induced ZENK gene expression within 10 cerebral regions and in parts of the cerebellum (**Figs. 3 and 4A, anterior and posterior regions; statistics in Table 1A**). Five of the 10 cerebral regions spanned the *anterior* portions of the five brain subdivisions that make up the medial half of the cerebrum and surrounded the AVP vocal nuclei in males. The other five cerebral regions made up a *posterior* forebrain pattern of activation, all laterally adjacent to the four PVP vocal nuclei in males and associated auditory fields (**Figs. 3 and 4A**).

Anterior Regions:

1. The anterior striatum (ASt) surrounding the vocal nucleus Area X (**Fig. 3Ac,d**)
2. The anterior nidopallium (AN) surrounding the vocal nucleus MAN (**Fig. 3Ac,d**)
3. The anterior ventral mesopallium (AMV) surrounding the vocal nucleus MO (**Fig. 3Ac,d**)
4. The anterior dorsal mesopallium (AMD; **Fig. 3Ac,d**)
5. The anterior hyperpallium apicale (AHA; **Fig. 3Ac,d**)

Posterior Regions:

6. The posterior lateral striatum (PLSt) lateral to the auditory field of CSt (**Fig. 3Ag,h**)

7. The posterior lateral nidopallium (PLN) lateral to vocal nucleus NIf and adjacent auditory fields L1 and L3 (**Fig. 3Ag,h**)

8. The posterior lateral ventral mesopallium (PLMV) lateral to vocal nucleus Av and auditory field CMM (**Fig. 3Ag,h**)

9. The dorsal lateral nidopallium (DLN) lateral and posteriorly adjacent to the vocal nucleus HVC and auditory HVC shelf (**Fig. 3Ag,h; 5A-C**)

10. The intermediate arcopallium (AI) laterally adjacent to song nucleus RA and the auditory RA cup (**Fig. 3Ag,h; 5D-F**).

The anterior activated regions (ASt, AN, AMV, AMD, and AHA) were located medially in the frontal half of the brain (**Fig. 3Ac,d**); they began with the AVP vocal nuclei medially and ended with them further laterally. We note here that MO is situated in the MV part of the mesopallium, as revealed by comparisons of GluR1 and ZENK expression (**Fig. 3Ad vs 3Ba**; also described further below for the zebra finch). For the posterior regions, the extent of the activation began with the lateral part of the PVP nuclei HVC and RA, and ended further laterally. DLN abutted the lateral-posterior part of vocal nucleus HVC and became larger further laterally, where HVC was not present (**Fig. 5A-C**). The activated region within AI also abutted the lateral part of vocal nucleus RA (**Fig. 5D-F**). PLN surrounded a strip of cells of no ZENK expression lateral to auditory L2, which also has no ZENK expression (**Fig. 3g,h vs 3c,d;** Mello and Clayton, 1994)). None of the four major vocal nuclei, AreaX, MAN, HVC, or RA, showed ZENK activation relative to birds sitting still (**Figs. 3 and 5**). As for the smaller vocal nuclei MO, NIf, and Av, they were more

difficult to locate due to their small size, but in the animals where they could be clearly identified, we did not note appreciable ZENK expression (**Fig. 3Ad** for MO; Supplementary **Fig. S1A** for NIf and Av). This is in contrast to the ZENK levels seen in these nuclei after singing (see below and Supplementary **Fig. S1B**; Jarvis and Nottebohm, 1997).

The exact pattern of gene expression varied between individuals and groups, as did the pattern of motor behavior. During the day time, garden warblers made more flights (549.4±135.8 [std. error] for day; 113.1±32.2 for night; t-test, $p<0.001$, $t=-4.659$) and in these birds, the posterior areas had higher activation relative to animals that performed MR during the night (**Fig. 4A, Table 1A**), and the anterior pattern of activation was more uniform throughout the respective brain subdivisions (**Fig. 3Ac vs 3Ad**). In the birds that performed MR, the part of AN directly caudal to the vocal nucleus MAN had the highest expression of all activated areas (**Fig. 3Ad & 4A**). For the cerebellum, in birds that performed MR, high ZENK activation was specific to lobules I-VI (**Fig. 6B**). For those that performed more flights, activation was more evenly distributed across lobules I-X (**Fig. 6C**) albeit a persisting difference between lobules VIb and IXa ($p<0.012$, $t=3.216$, Mann-Whitney). In general, the activation patterns in the cerebrum had a semi-columnar organization and of two types with parallel rostral and caudal boundaries: columns defined by St, N, and MV, and columns defined by HA and MD.

Correlation with the amount of movement

In all vocal learning birds, the amount of IEG activation in vocal nuclei is linearly proportional to the amount of vocalizations produced per 30-60 minutes and this has been a critical test to determine if the gene expression is motor-driven (Jarvis and Nottebohm, 1997; Jarvis et al., 1998; Jarvis and Mello, 2000; Jarvis et al., 2000). To test for this

property in the defined regions of this study, we performed correlation analyses on the amount of gene expression with the amount of movement. We plotted the level of gene expression against the number of wing beats, as wing beats made up the main part of garden warbler motor activity. The wing beat count includes both those performed while perched and in flight. They ranged in different birds from 0 to 7409 in 60 minutes, including day time and night time groups. This analysis resulted in statistically significant polynomial 2nd order regression relationships between wing beats and gene expression for all 11 brain areas (**Fig. 4B, Table 1B**). ZENK expression levels reached a maximum at around 6000 wing beats and then saturated, and may have even started to decrease (or perhaps habituate; Mello et al., 1995) in some birds. The correlations were strongest for the regions of the anterior forebrain column ASt-AN-AMV and the cerebellum.

Although the expression relationships we measured were significantly correlated with wing beats, there was still obvious scatter of expression values. Thus, we asked whether the relationships were any tighter with the number of flights or with the number of head scans. Regressions with flights or head scans alone were significant but weaker than with wing beats. Of these two categories, the strongest relationship with flights was found in AHA ($r=0.700$; $p<0.001$) followed by AMD ($r=0.681$; $p<0.001$; **Table 1B**) – consistent with the more uniform pattern of expression in AHA of birds that flew a lot (**Fig. 3Ac**). The strongest relationship with head scans was found in AN directly caudal to MAN ($r=0.664$; $p<0.001$) followed by ASt caudal to AreaX ($r=0.602$; $p=0.002$) – consistent with AN being the area of highest activation in birds performing MR in the night in connection with head scan movements (other values for head scans and quantifications in other parts of ASt and AN not shown). Thus, the areas we identified may be preferentially activated during different movement types and/or movements of different body parts, and this could create the scatter of values.

Non-motor related sensory behaviors

In these 11 brain regions, there was no induction due to sensory stimulation by day- or dim-light (**Fig. 4A, compare sitting day still and night still birds, Table 1A**). To determine whether the movement related induction was specific to these brain regions or reflected a general increase in brain activation in moving birds, we analyzed ZENK expression in six regions known to show ZENK induction following various sensory tasks or stimuli: the hippocampus (Hp), which shows IEG activation induced by spatial tasks, e.g. food-hoarding (Smulders and DeVoogd, 2000); NCM, a higher order auditory processing region, activated upon hearing species-specific songs (Mello et al., 1992); L3, a lower order auditory region activated upon hearing (Theunissen et al., 2004); ALN and ALMV immediately anterior to the entopallium (E, a visual area), activated by day-light vision (Mouritsen et al., 2005); and cluster N (a HA-MD column), activated by dim-light, night-vision in migratory songbirds (Mouritsen et al., 2005).

None of these six brain regions showed the activation pattern seen in the 11 movement related brain regions (**Fig. 4A, other brain areas**). Instead, the Hp and NCM showed no differences among groups (**Figs. 3 and 4A, Table 1A**). L3 (as well as L1 examined qualitatively) showed high ZENK expression only during MR behavior (**Figs. 3Ad and 4A, night-time movement group**), and no induction during day time movement (**Figs 3Ac and 4A**). We surmise that L3 (and L1), which processes simple sounds, may be responding to the self-produced wing whirring sounds of MR behavior; in support of this idea, L3 expression had a significant correlation with the number of wing beats but not with the number of flights made (**Table 1B**). ALN and ALMV showed strong induced ZENK expression by day light (**Figs. 3Ae,g and 4A; Table 1A, sitting still birds**). There was a secondary weak relationship with movement (**Figs. 4A, Table 1A**). We surmise that

ALN and ALMV, which receives input from the entopallium that is involved in visual motion perception (Nguyen et al., 2004), may be responding to optic flow in both day and night moving birds; in support of this idea, ALN and ALMV had a significant correlation with the number of flights made but not with the number of wing beats (**Table 1B**); flights will cause optic flow, but wing whirring while sitting will not. Cluster N showed strong induced expression by dim light and had no noticeable relationship with movement (**Figs. 3Af,h and 4A**). We also examined the entopallium (E) as well as the globus pallidus (GP), two areas known not to express ZENK (Mello and Clayton, 1995); but GP is involved in motor behavior (Reiner et al., 2004a). We found a *very small* quantitative increase in the day time animals (**Fig. 4A, Table 1A**) suggesting that this region may in fact express some ZENK at a barely detectable level, but there was no overall difference in moving animals. We conclude that high ZENK activation in the 11 brain regions in moving animals is specific to these areas and not the result of a general increase in brain ZENK expression.

To verify the functional and anatomical relationships with an independent statistical measure, we performed principal component analysis (PCA) on all quantified brain regions together. The PCA nicely separated the brain regions and revealed additional information. Component 1 separated the movement-induced regions from all other regions (**Fig. 7**). Component 2 further split the movement activated regions into distinct clusters: an anterior cluster, tightest for ASt, AN, AMV (adjacent to AVP vocal nuclei) along with the Cb; a posterior cluster consisting of PLSt, PLN, and PLMV; and a posterior cluster consisting of DLN and AI (adjacent to HVC and RA, respectively). Component 3 separated the non-movement related areas among each other, but keeping ALN and ALMV, day activated regions, together as a cluster. This clustering of different regions together across adjacent brain subdivisions, as opposed to different regions from the same brain subdivision, supports the columnar organization noted earlier.

We noticed that some areas in the brains of our animals showed high ZENK expression not related to any specific group. These included, but were not limited to the lateral striatum, which had high expression in all groups, and the anterior and posterior parts of the arcopallium, which had high expression in some birds of all groups (**Fig 3Ae-h;** quantifications not shown). Perhaps activation in these areas is associated with being awake, or simply standing. Nevertheless, activation was not specific to the movement groups, lending further support that the activation pattern of the 11 brain regions is specific to movement.

A non-migratory songbird

We analyzed brains of zebra finches, a non-migratory songbird species, that were placed in our behavior apparatus. Zebra finches were highly mobile during the day where they ran and jumped around in the cage. The ZENK expression patterns in moving day time finches were similar to the ones found in the moving day time group of warblers, except that in zebra finches that mainly ran around in the cage, the activation of the anterior ASt-AN-AMV column was more narrowly focused around the AVP nuclei (**Fig. 6B,E**). Activation in the AMD-AHA column still spanned the caudal to rostral extent of these brain regions. There were scattered cells with high expression in some vocal nuclei (AreaX and MO; **Fig. 6B**), but this paled in comparison to the high activation induced in the vocal nuclei by singing (**Fig. 6C**). For the cerebellum, a gradient of higher expression was concentrated towards lobule VI as in garden warblers, but there was one interesting deviation: a zebra finch that constantly flipped its head side-to-side and up-and-down showed higher activation across lobules VII-VIII (**Fig. 6F**). There was not enough variation in running and hopping behavior of the zebra finches to test for correlations. Taken together, these

results show that movement associated ZENK activation within the described 11 brain regions is not specific to garden warblers or MR behavior, but is more general.

Females: vocal non-learners

Females of many songbird species do not have vocal learning behavior, i.e. song, and have atrophied vocal nuclei (except for LMAN) in their forebrain. This is the case for zebra finches (Nixdorf-Bergweiler, 1996) and for garden warblers (noted here for atrophied vocal nuclei). We separated out the females in the groups described above, and found that in the female zebra finch (n=1) and garden warblers (n=6) that performed movement behavior (running, wing beats, flights, and/or head scans) ZENK gene activation was present within the same 10 cerebral regions and in the cerebellum as seen in males (n=4 finches and 14 warblers), but without the presence of negative expression regions of vocal nuclei (**Fig. 9,** except for LMAN). Thus, these movement-activated brain regions appear to be present independent of the vocal nuclei.

Discussion

This is the first study that we are aware of to functionally identify non-vocal motor brain areas in birds. We found that cerebral areas that show motor-driven gene expression during movement behavior surround or are adjacent to the vocal nuclei that show motor-driven gene expression by singing. The anatomical extent of the non-vocal motor areas is much larger than that of the vocal nuclei, which is consistent with a much greater amount of musculature involved in controlling head, wing, and body movements compared to that necessary for controlling the syrinx. Our behavioral approach, however, was not specific enough to map a possible homunculus organization as seen in the mammalian motor cortex

(Penfield and Rasmussen, 1957), or motor versus premotor distinctions. Below we discuss the implications of our results for understanding motor pathways in birds and the evolution of brain pathways for vocal learning.

General implications for future studies using IEG expression

Since it is difficult to make birds sit absolutely still during a sensory task and obviously impossible during behavioral tasks, general motor activation has the potential to distract experimenters' attention from differences in more relevant brain areas. Thus, knowing the brain regions controlling motor behavior will be important for future studies using behavioral molecular mapping to identify brain areas involved in specific behaviors and/or sensory learning tasks. Future experimenters can now exclude the motor activated areas from their analyses or perform correlations of the gene expression with the amount of movement of their birds. Conversely, the discovery of the functional gene expression activation of these brain areas will now allow investigators to probe specific sites in the avian central nervous system for control of movement.

Motor Pathways in Birds

The activation patterns in the cerebrum were organized in a semi-columnar fashion, with parallel rostral and caudal boundaries. The anterior-medial St-N-MV column (ASt-AN-AM) mirrored a singing-driven gene expression column consisting of AreaX, MAN, MO, i.e. the AVP vocal nuclei. A posterior-lateral St-N-MV column consisted of PLSt-PLN-PLMV, situated lateral to the PVP vocal nuclei. The only non-columnar activation was found in DLN and AI, lateral to song nuclei HVC and RA that are also not organized as a column. Both the vocal and non-vocal motor patterns suggest that much like the functional columnar organization of mammalian cortical layers and associated striatum,

brain systems in the avian cerebrum may have a functional columnar organization sculpting out portions of each brain subdivision: striatum, nidopallium, mesopallium, hyperpallium, and arcopallium.

Interestingly, the connectivity of the AVP vocal nuclei in songbirds (**Fig. 10A**; and in parrots) is comparable to the corresponding regions around them (Durand et al., 1997; Iyengar et al., 1999; Farries, 2001; Diekamp and Gunturkun, 2003). These corresponding regions are connected in a pallial-basal-ganglia-thalamic-pallial loop (**Fig. 10;** white arrows): anterior mesopallium (formerly hyperpallium ventrale) to anterior nidopallium (formerly neostriatum), these two regions to the striatum (formerly LPO), the striatum via pallidal-like neurons to the dorsal thalamus and the dorsal thalamus back to the anterior nidopallium (connectivity of MO or the surrounding MV is not known in songbirds, but in parrots projects to the striatum (Durand et al., 1997)). Thus, we suggest that the anterior-medial portion of the avian forebrain forms a general anterior motor pathway (AMP; or more literally a premotor pathway) for motor behavior in birds. Our results do not indicate whether this AMP is involved in motor learning, as is the AVP, but like the AVP it is active in production of motor behavior.

The connectivity of the PVP vocal nuclei may also be similar to the connectivity among several of the posterior movement activated brain areas (**Fig. 10; black arrows**). DLN is situated in the dorsal lateral nidopallium (NCL), which has connections similar to songbird HVC (Margoliash et al., 1994; Iyengar et al., 1999; Farries, 2001). The NCL receives input from the areas around and between LMAN and MMAN in songbirds, and from the comparable region in non-songbirds; it projects to the AI, which in turn projects to brainstem premotor and motor neurons. The shell around LMAN also projects to AI, in a similar manner as LMAN projects to RA (Iyengar et al., 1999). It is not possible to determine definitively whether the DLN and the part of AI we identified in this study are

exactly the regions that receive input from around MAN and connect with each other. However, since the general NCL region receives input from the anterior nidopallium and projects to the arcopallium, it is likely that the motor-activated DLN has the same connectivity. Taken together, the expression patterns found here and the connectivity known from other studies suggest a non-vocal posterior motor pathway (PMP) working in parallel to the PVP.

We also found activation within three brain subdivisions that do not have vocal nuclei – the AHA, the AMD adjacent to it, and the cerebellum. Activation in these brain regions during movement may be somatosensory related. The AHA has been proposed to be somatosensory pallium, as it receives substantial somatosensory input and shows neural firing to somatosensory stimulation along the body and limbs (Wild, 1997b; Wild and Williams, 2000a). Some of the heaviest input into AHA comes from wing somatosensory pathways (Wild and Williams, 2000a), consistent with our finding of higher correlation of gene activation with flights. AHA also receives projections from AMD, but the source of inputs to AMD is not well known (Wild and Williams, 2000a). Interestingly, in songbirds, AHA sends some of its heaviest cerebral projections to the areas around the AVP vocal nuclei, to the NCL lateral to HVC, and AI (Wild and Williams, 1999) – all in a pattern strikingly similar to the movement related gene expression we found here in zebra finches (**Fig. 10**). In female songbirds, the AHA cerebral projection pattern is similar to the movement related gene expression pattern we found without vocal nuclei (Wild and Williams, 1999). This overlap of connectivity and gene expression patterns suggests that AHA may transmit somatosensory input into anterior and posterior motor pathways adjacent to vocal nuclei, and perhaps modulate their activity.

Like AHA, the cerebellum also receives somatosensory input to modulate motor neurons for fine coordination of movements (Wild and Williams, 2000b; Necker, 2001). It

was in the cerebellar granule layer where we found the high levels of movement-related ZENK expression. This granule layer projects to the molecular layer and from there onto the purkinje neurons, which then send out motor commands. We did not see activation in these other neuron populations. Presumably they do not have the necessary receptors for activity-induced ZENK expression (Jarvis, 2004a; Wada et al., 2004), and/or may be silenced by DNA methylation at cis-regulatory promoter elements. The granule layer of the cerebellum lobules in birds have two somatotopic body representations: one located in the anterior half of the cerebellum from lobules I-VI and the other located in the posterior half from lobules IX-X (Necker, 2001). Lobules VII and VIII receive input from the pallium, including AHA (Wild and Williams, 2000b). In the anterior half, lobules I-III receive input from the neck, III-V from the wing, and VI from the legs (with some input from the legs also to III-V) (Necker, 2001). This anterior half showed preferentially higher activation during MR movement, consistent with lobules III-VI responsible for wings and legs that are intensely used during MR. Activation was more uniform throughout the lobules when flights were preferentially made, consistent with the idea that flights involve the utilization of more body musculature than wing whirring while perched.

Given these findings, we hypothesize that during movement, a posterior and anterior motor system consisting of columns and components of St-N-MV and A control the production and sequencing of movements, and that a cerebral somatosensory system consisting of a medial HA-MD column provides somatosensory feedback to the anterior and posterior motor systems and the cerebellum. The more a movement is performed, the more the motor and also somatosensory activity causes increased neural activity-dependent gene expression in the controlling brain regions. The patchiness of the cerebral activation patterns in different animals performing predominantly different movements suggests that there are parallel motor pathways for either different movements or muscles groups. This

hypothesis and our related findings have implications for understanding the evolution of vocal learning.

Evolution of vocal learning

Seven of the 10 cerebral brain regions active in movement behavior are adjacent to or surround the well-known songbird PVP and AVP vocal nuclei. Thus, our functional results combined with connectivity findings (**Fig. 10**) suggest that the vocal learning system of songbirds (and perhaps of distantly related vocal learning birds) evolved out of a pre-existing motor system that controls learning and producing non-vocal motor behavior (Farries, 2001; Jarvis, 2004b). If true, then only one type of major connection is missing in vocal non-learners – from the arcopallium to the DM premotor and nXIIts vocal motor neurons of the brainstem (**Fig. 10A, red arrows**; Wild, 1997a; Jarvis, 2004b). If during evolution, such connections are formed through mutation then it may be possible to usurp a pre-existing motor cerebral pathway for the cerebral control of vocal motor behavior. The part of the pathway that could be exploited may determine the location of the AVP nuclei, which is quite similar among the different vocal learners (**Fig. 1**). This suggests an important anatomical constraint. This constraint may be the location of neurons that control head movements, as head scanning showed the highest activation relationship with the brain regions posteriorly adjacent to the AVP nuclei. Perhaps in order to evolve vocal learning, it is necessary to usurp the circuit synapsing onto muscles close to the syrinx – i.e. head and neck muscles. The location of the posterior motor activated areas lateral to auditory regions may also place them in an opportunistic position to take advantage of auditory input for evolving a vocal motor learning system, which requires auditory-related sensorimotor feedback.

Vocal learning also exists in some distantly related mammals (humans, bats, cetaceans, and elephants (Reiss and McCowan, 1993; Esser, 1994; Poole et al., 2005). For humans, recent analyses indicate that cerebral vocal regions are adjacent to or embedded in the motor and premotor brain areas (Lieberman, 2002; Jarvis, 2004b). This has been supported by recent findings showing that the premotor part of that system, Broca's area, controls oral-facial (but not laryngeal) movements in a non-human primate, macaques, a vocal non-learner (Petrides et al., 2005). Thus, the evolution of vocal learning brain systems out of a pre-existing motor system could well be a general feature of the vertebrate brain.

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Figure Legends

Figure 1. Phylogenetic relationships and brains of avian vocal learners. *(A)* One view of phylogenetic relationship of living birds (Sibley and Ahlquist, 1990), with vocal learners highlighted in red, possible independent gains of vocal learning highlighted with red dots, or possible independent losses highlighted with green dots. *(B)* Semi-3D view of seven cerebral vocal regions (yellow and red in cerebrum) found in each vocal learner, and of auditory regions (blue) found in all birds. Red-labeled vocal nuclei and white arrows: *anterior vocal pathway (AVP)*. Yellow-labeled vocal nuclei and black arrows: *posterior vocal pathway (PVP)*. Only a few connections in hummingbirds are known and that of songbird MO is not known. Scale bars: 1 mm. Figure modified from Jarvis et al. (2000) and Jarvis, (2004b). Brain area abbreviations are found in the legend of figure 3.

Figure 2. Experimental behavior apparatus. The apparatus is cylindrical, made of transparent Plexiglas on the sides and bottom, a removable net at the top, and a round black perch at the center of the floor. Infrared-emitting video-cameras (*IR*) attached to video monitors and recording equipment are used to observe and record the bird's behavior. Reproduced with permission from Mouritsen et al. (2004a).

Figure 3. Movement activated brain areas. (*Part A and B of the figure are to on separate adjacent pages) (A)* Representative darkfield images of ZENK expression in medial *(a-d)* and lateral *(e-h)* sagittal sections of the four main garden warbler groups studied. Dorsal is up, anterior is right. White silver grains: ZENK expression. Red: nissl stain. *(B)* GluR1 expression pattern used along with Nissl staining to localize brain subdivision boundaries. The GluR1 expression pattern shows that MD and MV are part of a single structure, the

mesopallium (Reiner et al., 2004b). Camera lucida drawings are to the right. Red outlines: extent of movement activated regions. Green outlines: day vision activated regions. Blue outlines: night vision activated regions. The abbreviations are placed in the center of quantified regions shown in figure 4. Scale bar, 2 mm.

Abbreviations:

A, arcopallium; AAc, central nucleus of the anterior arcopallium; ACM, caudal medial arcopallium; AHA, anterior hyperpallium apicale; AI, intermediate arcopallium; AMD, anterior dorsal mesopallium; AMV, anterior ventral mesopallium; AN, anterior nidopallium; ASt, anterior striatum; Av, avalanche; Cb, cerebellum with granular (*gr*), purkinje (*p*) and molecular (*mol*) cell layers; CMM, caudal medial mesopallium; CSt, caudal striatum; DLN, dorsal lateral nidopallium; DM, dorsal medial nucleus of the midbrain; DMm, magnocellular nucleus of the dorsomedial thalamus; E, entopallium; GP, globus pallidus; HA, hyperpallium apicale; Hp, hippocampus; ICo, inferior colliculus; IHA, intercalated hyperpallium apicale; LAN, lateral nucleus of the anterior nidopallium; LAM; lateral nucleus of the anterior mesopallium; M, mesopallium; MAN, magnocellular nucleus of the anterior nidopallium; MLd, dorsal part of the lateral mesencephalic nucleus; MMSt, magnocellular nucleus of the medial striatum; MO, oval nucleus of the mesopallium; MD; dorsal mesopallium; MV, ventral mesopallium; N, nidopallium; NAOc, oval nucleus complex of the anterior nidopallium; NIDL, dorsal lateral intermediate nidopallium, NIf, interfacial nucleus of the nidopallium; NLc, central nucleus of the lateral nidopallium; nXIIts, 12th nucleus tracheosyringeal part; PLMV, posterior lateral ventral mesopallium; PLN, posterior lateral nidopallium; PLSt, posterior lateral striatum; RA, robust nucleus of A; S, septum; St, striatum; v, ventricle; VA, vocal nucleus of the arcopallium; VAM, vocal nucleus of the anterior mesopallium; VAN, vocal nucleus of the

anterior nidopallium; VASt, vocal nucleus of the anterior striatum; VLN, vocal nucleus of the lateral nidopallium.

Figure 4. Quantification of ZENK expression in garden warbler brains. *(A)* ZENK expression levels (image pixel density) in the four groups of birds, in 11 brain regions with movement-induced gene expression (*anterior, posterior*, and *Cb [lobule VI] regions*) and in other brain regions that do not show expression specifically related to movement (*other*). The absolute cluster N (ClN) values are different from our previous report (Mouritsen et al., 2005), because we did not use other brain regions as a normalization factor in the present study. Error bars: SE. *(B)* Example regression graphs for brain regions that show ZENK expression levels correlated (ASt, AN, AMV) and not correlated (Hp) with movement performed within the last 60 min. Statistics for *(A)* and *(B)* are in Table 1A and 1B, respectively. Brain area abbreviations are in the legend of figure 3.

Figure 5. Movement-driven induced gene expression adjacent to vocal nuclei HVC and RA. *(A-C)* Medial-to-lateral series, beginning lateral to HVC of Figure 3Ad, showing that region of DLN expression (white arrows) becomes larger the further lateral from HVC (black arrows); brain sections are from a garden warbler that performed MR behavior in the night. *(D-F)* Medial-to-lateral series showing AI expression (white arrows) lateral to song nucleus RA (black arrows); sections are from a garden warbler that performed flights during the day. The HVC shelf expression in *(A)* was not always seen further medially, and we do not know how much of this is movement (this study) or hearing related (Mello and Clayton, 1994; Jarvis and Nottebohm, 1997). The dark spot in DLN of panel *(C)* is torn tissue. The arcopallium (including RA; Jarvis and Nottebohm, 1997) generally has less

induced ZENK expression in its core region than do other brain subdivisions. Dashed line in E shows the boundary of the arcopallium with the nidopallium dorsal to it and striatum anterior to it. Sections are sagittal; dorsal is up, anterior is right. Scale bar, 0.5 mm.

Figure 6. Higher power view of ZENK expression in the cerebellum of garden warblers that *(A)* sat relatively still for 60 min during the night, making only one flight, *(B)* that performed MR behavior for 60 min during the night, and *(C)* that made flights and moved around in the cage during the day. Lobule numbering follows Necker (2001). Scale bar, 1 mm.

Figure 7. ZENK expression in zebra finch brains. Shown are brain sections of anterior regions of *(A)* a bird sitting still, *(B)* running around the cylindrical cage, and *(C)* singing; there is some self-hearing induced expression in L3 and L1 around L2 and in CMM posterior to AMV. *(D)* Quantification of ZENK expression levels in anterior regions (ASt, AN, AMV) around AVP vocal nuclei that shows movement induced expression and a control region (Hp). Error bars: SE. *(E)* Anatomical profile of the brain section shown in *(B)*. *(F)* High power image of the cerebellum of a zebra finch that repetitively flipped its head, resulting in exceptionally high ZENK expression in the transition zone of lobules VII to VIII. All in-situ images are sagittal sections; rostral right, dorsal up. Scale bar in *(A-C)* 2 mm; in *(F)* 0.5 mm. Brain area abbreviations are in the legend of figure 3.

Figure 8. Principal component analysis. Two views are shown: the left shows component 1 more clearly; the right shows component 2 more clearly. Values from each brain region are plotted according to the first three principal components. They are color-coded

according to clustering with nearest neighbors in the graph. Brain area abbreviations are in the legend of figure 3.

Figure 9. Movement-driven ZENK expression in males versus females. (*A)* Male and (*B)* female garden warbler sections, at a high power view of the anterior region of activation. The male AreaX and MAN have very low expression, and male MO and female MAN is slightly higher. A central column in this female's striatum has a gradient of lower expression, and the dorsal part of it we speculate could be remnants of AreaX or simply part of the pattern of movement-driven gene expression dependent upon the type of movements performed. Dorsal is up, anterior is right. Scale bar: 0.5mm. Brain area abbreviations are in the legend of figure 3.

Figure 10. Schematic drawing of vocal motor pathway *(A)* and the hypothesized non-vocal motor pathway *(B)*. Motor activated brain areas of the anterior forebrain surrounding the AVP vocal nuclei (AreaX, MAN, and MO) in *(B)* are defined at the minimum size seen in this study. Motor activated areas adjacent to PVP vocal nuclei (HVC, RA, NIf, and Av) in *(B)* are defined at their average size, and with dashed lines, as they are situated lateral to the plane of section shown. White arrows: connectivity of AVP pathway *(A)* and of brain areas immediately outside of the AVP vocal nuclei in songbirds *(B)*. Black arrows: connectivity of PVP pathway *(A)* and of brain areas outside of the PVP vocal nuclei of songbirds *(B)*. The compiled connectivity findings for the non-vocal areas are derived form the studies mostly in (Margoliash et al., 1994; Wild et al., 1997; Iyengar et al., 1999; Bottjer et al., 2000) and also compiled in (Farries, 2001; Jarvis, 2004b). The projection of AMV to the striatum is proposed based upon findings in parrot (Durand et al., 1997) and pigeon (Veenman et al., 1995). The connection from MAN to HVC and RA is generally

shown; specifically medial MAN projects to HVC and lateral MAN to RA. Abbreviations. DMP, dorsal medial nucleus of the posterior thalamus; MN, motor neurons; NCL, caudal lateral nidopallium; PMN; premotor neurons; UVa, nucleus uvaformis. Remaining abbreviations are in the legend of figure 3.

Supplementary Figure S1. Further assessment of motor-driven ZENK expression in and outside of vocal nuclei. *(A)* High power views of NIf and Av from a garden warbler that performed MR movement behavior (same animal as in Figure 3Ac). Note sharp columnar boundary of expression anterior to NIf that crosses brain subdivision boundaries of the striatum, nidopallium, and mesopallium. Scale bar: 0.5 mm. *(B)* Brain section of zebra finch that sang showing higher activation in HVC, NIf, Av, AreaX, MAN, and MO. Scale bar: 2 mm. Brain area abbreviations are in the legend of figure 3.

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Table 1: Statistical analysis. *(A)* Results of one-way ANOVA followed by Holm-Sidak all-pairwise multicomparison test for all brain regions quantified in Figure 4A. *(B)* Results of 2nd order polynomial regression, for all brains regions quantified, including for the graphs in Figure 4B. Significant differences are highlighted with red text.

brain area		ASt	AN	AMV	AMD	AHA	PLSt	PLN	PLMV	DLN	AI	Cb	Hp	NCM	L3	ALN	ALMV	Cluster N	E	GP
A. ANOVA	p-value	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.243	0.064	0.001	0.001	0.001	0.001	0.208	0.053
post-hoc test	day_{still} $night_{still}$	0.853	0.269	0.823	0.593	0.476	0.760	0.516	0.536	0.567	0.075	0.744			0.841	0.001	0.001	0.001		
	day_{still} day_{act}	0.001	0.001	0.007	0.019	0.007	0.003	0.002	0.002	0.001	0.029	0.029			0.852	0.022	0.035	0.314		
	$night_{still}$ $night_{act}$	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.018	0.004	0.001	0.001			0.001	0.019	0.043	0.111		
	day_{act} $night_{act}$	0.995	0.347	0.990	0.790	0.579	0.152	0.052	0.006	0.001	0.065	0.763			0.006	0.001	0.001	0.001		
B. polynomial	wing beats R=	0.821	0.834	0.712	0.738	0.723	0.641	0.654	0.560	0.638	0.618	0.777	0.076	0.111	0.498	0.337	0.305	0.244	0.049	0.344
	wing beats p<	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.911	0.826	0.012	0.136	0.210	0.363	0.961	0.125
	flights R=	0.575	0.612	0.653	0.681	0.700	0.678	0.655	0.632	0.631	0.624	0.624	0.228	0.235	0.297	0.516	0.467	0.399	0.159	0.366
	flights p<	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.426	0.415	0.238	0.006	0.020	0.057	0.654	0.093

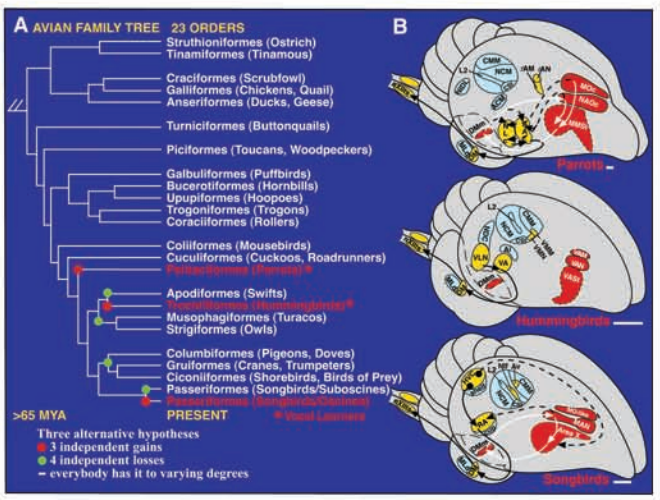
A AVIAN FAMILY TREE 23 ORDERS
Struthioniformes (Ostrich)
Tinamiformes (Tinamous)
Craciformes (Scrubfowl)
Galliformes (Chickens, Quail)
Anseriformes (Ducks, Geese)
Turniciformes (Buttonquails)
Piciformes (Toucans, Woodpeckers)
Galbuliformes (Puffbirds)
Bucerotiformes (Hornbills)
Upupiformes (Hoopoes)
Trogoniformes (Trogons)
Coraciformes (Rollers)
Coliiformes (Mousebirds)
Cuculiformes (Cuckoos, Roadrunners)
Psittaciformes (Parrots)*
Apodiformes (Swifts)
Trochiliformes (Hummingbirds)*
Musophagiformes (Turacos)
Strigiformes (Owls)
Columbiformes (Pigeons, Doves)
Gruiformes (Cranes, Trumpeters)
Ciconiiformes (Shorebirds, Birds of Prey)
Passeriformes (Songbirds/Suboscines)
Passeriformes (Songbirds/Oscines)*
>65 MYA
PRESENT
* Vocal Learners
Three alternative hypotheses
3 independent gains
4 independent losses
= everybody has it to varying degrees
B
Parrots
Hummingbirds
Songbirds

Figure 1

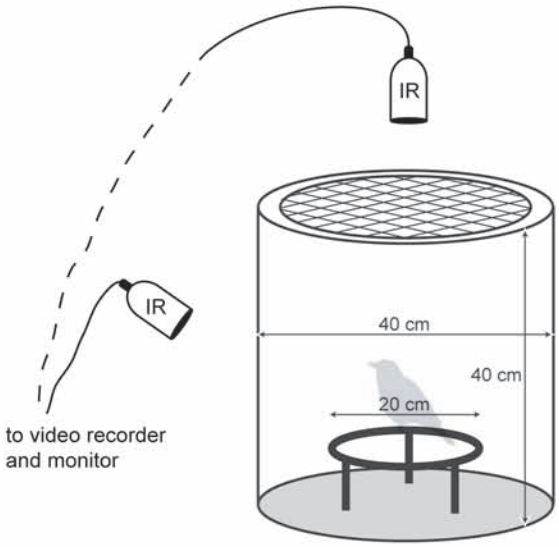

Figure 2

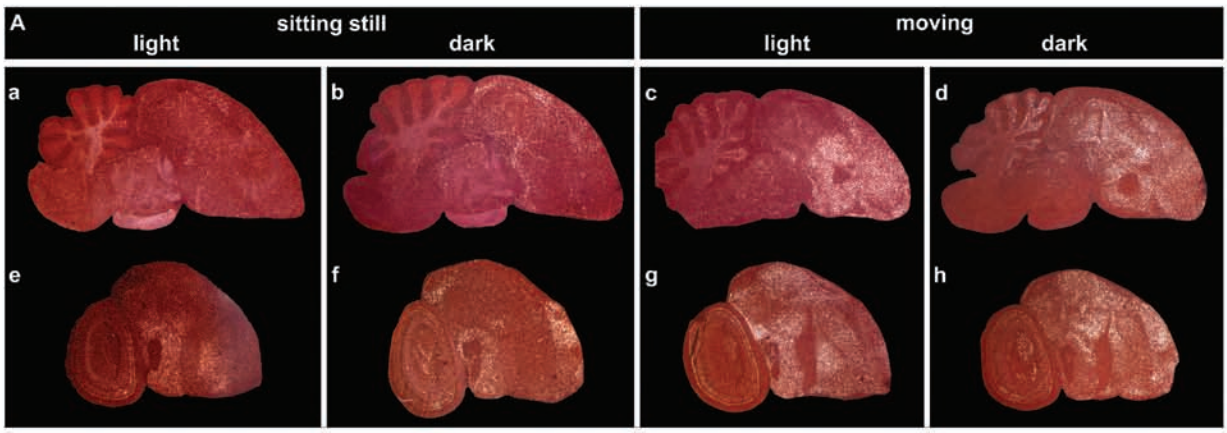

Figure 3A

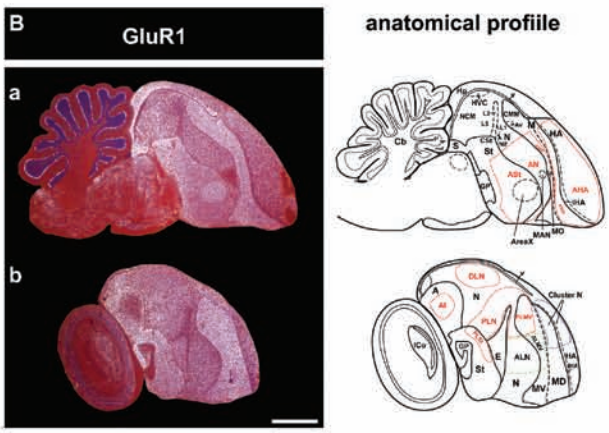
B
GluR1
a
b
anatomical profiile
Cb
St
HA
MAN
AreaX
MO
DLN
N
Cluster N
PLN
ALN
MD
MV

Figure 3B

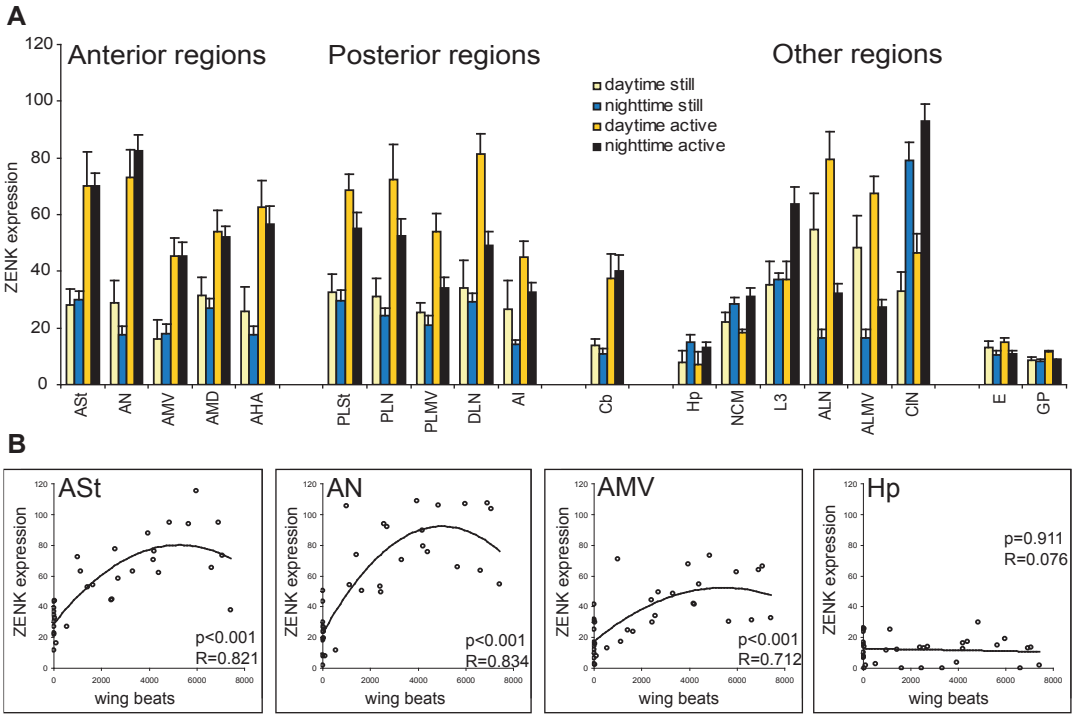
A
Anterior regions
Posterior regions
Other regions
daytime still
nighttime still
daytime active
nighttime active
ZENK expression
ASt
AN
AMV
AMD
AHA
PLSt
PLN
PLMV
DLN
AI
Cb
Hp
NCM
L3
ALN
ALMV
CIN
E
GP
B
ASt
p<0.001
R=0.821
AN
p<0.001
R=0.834
AMV
p<0.001
R=0.712
Hp
p=0.911
R=0.076
wing beats

Figure 4

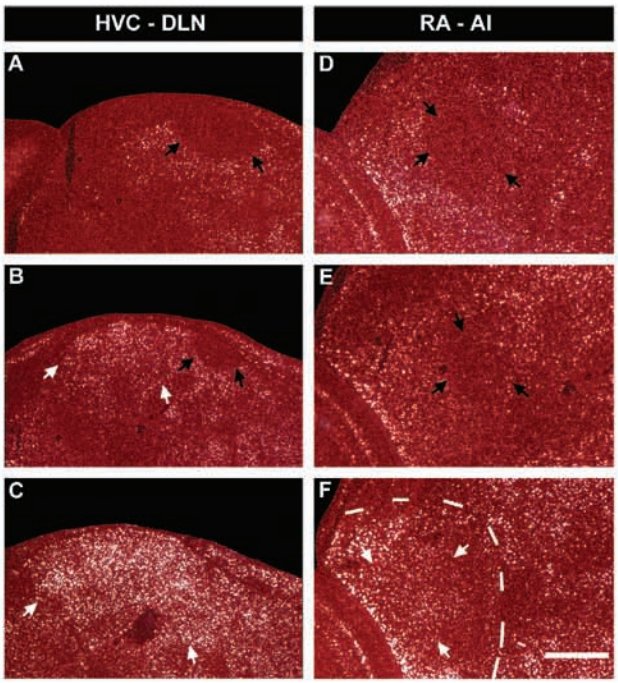

Figure 5

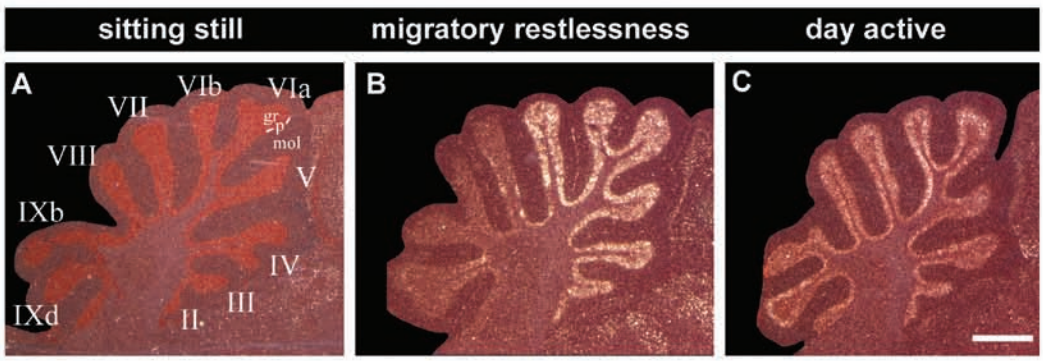
sitting still
migratory restlessness
day active
A
VII
VIb
VIa
gr
p'
mol
VIII
V
IXb
IV
III
IXd
II
B
C

Figure 6

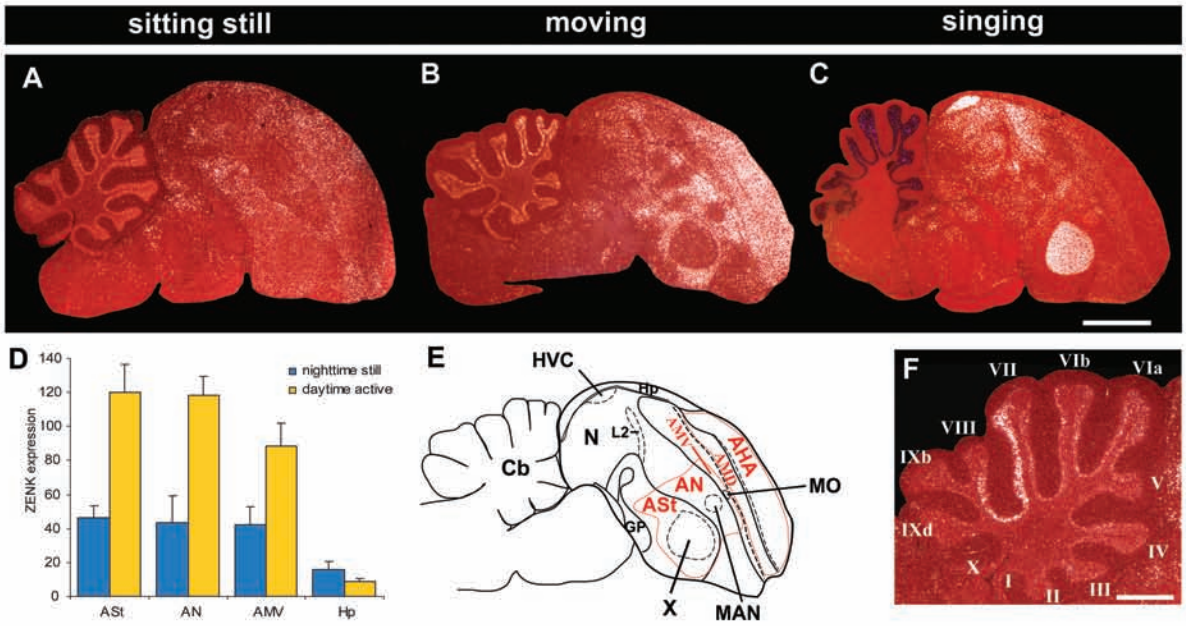
sitting still
moving
singing
A
B
C
D
ZENK expression
nighttime still
daytime active
ASt
AN
AMV
Hp
E
HVC
Hp
N
L2
Cb
AMV
AHA
AN
MO
ASt
GP
X
MAN
F
VII
VIb
VIa
VIII
IXb
V
IXd
IV
X
I
II
III

Figure 7

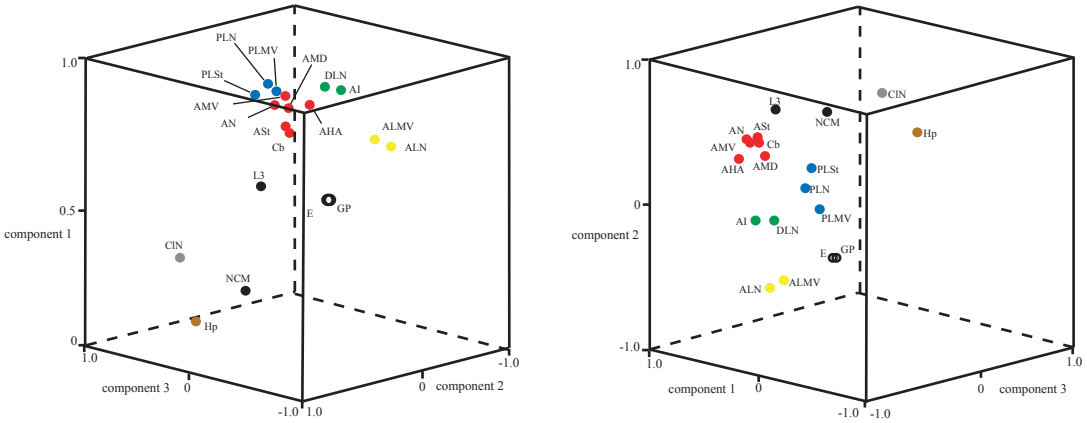
PLN
PLMV
AMD
DLN
AI
PLSt
AMV
AN
ASt
AHA
ALMV
Cb
ALN
L3
E
GP
ClN
NCM
Hp
1.0
0.5
0
component 1
1.0
component 3
0
-1.0
1.0
0
component 2
-1.0
1.0
component 1
0
-1.0
1.0
component 3
-1.0
1.0
component 2
0
-1.0

Figure 8

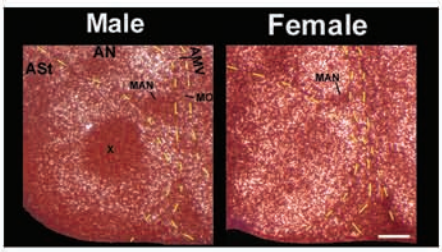

Figure 9

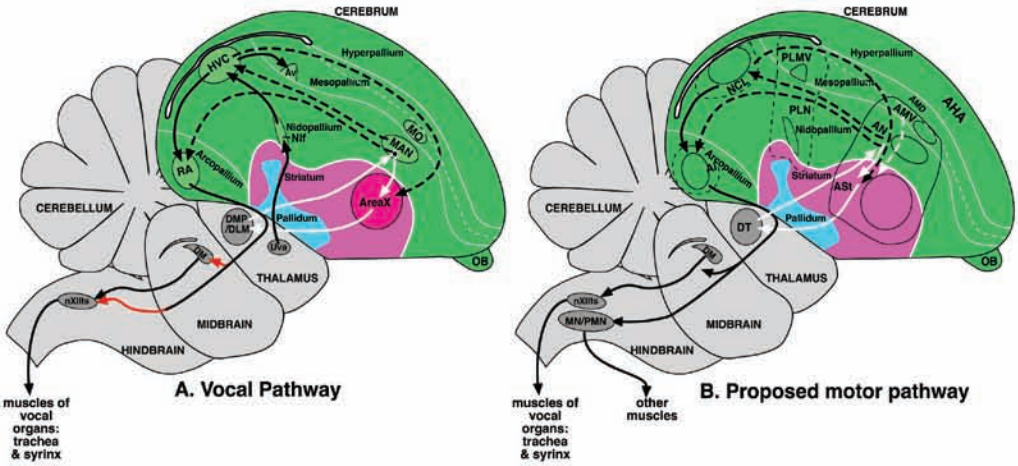
CEREBRUM
Hyperpallium
HVC
Av
Mesopallium
Nidopallium
NIf
MO
MAN
RA
Arcopallium
Striatum
AreaX
CEREBELLUM
DMP
/DLM
Pallidum
Uva
OB
THALAMUS
nXIIts
MIDBRAIN
HINDBRAIN
A. Vocal Pathway
muscles of
vocal
organs:
trachea
& syrinx
CEREBRUM
Hyperpallium
PLMV
NCL
Mesopallium
AHA
AMV
PLN
Nidopallium
AN
Arcopallium
Striatum
ASt
CEREBELLUM
DT
Pallidum
OB
THALAMUS
nXIIts
MN/PMN
MIDBRAIN
HINDBRAIN
B. Proposed motor pathway
muscles of
vocal
organs:
trachea
& syrinx
other
muscles

Figure 10

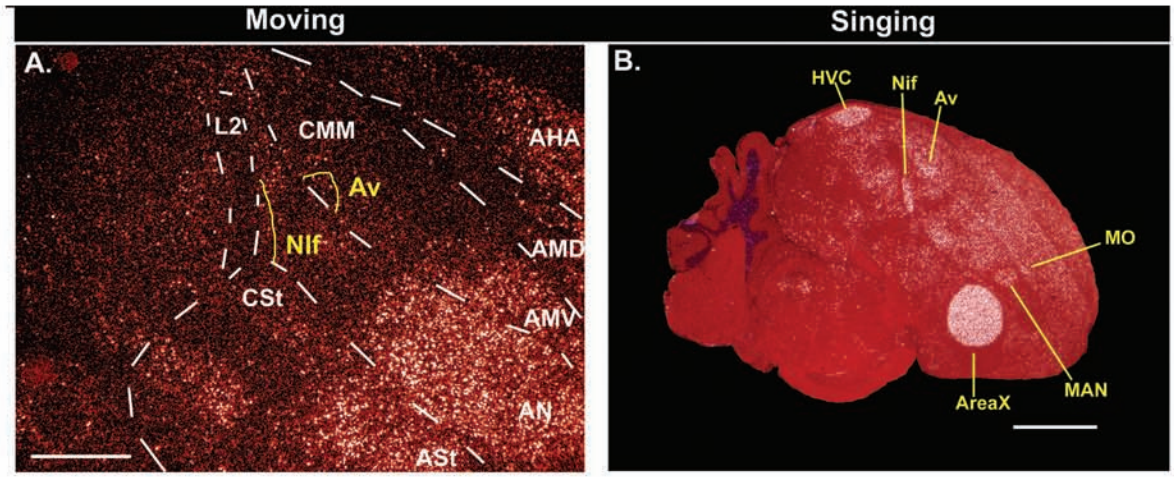
Moving
Singing
A.
L2
CMM
AHA
Av
Nif
AMD
CSt
AMV
AN
ASt
B.
HVC
Nif
Av
MO
AreaX
MAN

Figure S1

Erklärung

Hiermit erkläre ich, dass ich diese Arbeit selbstständig angefertigt und nur die angegebenen Hilfsmittel verwendet habe. Die vorliegende Dissertation hat weder in ihrer Gesamtheit noch in Teilen einer anderen wissenschaftlichen Hochschule zur Begutachtung in einem Promotionsverfahren vorgelegen.

Oldenburg, 18. Juli 2006

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Miriam Liedvogel