Response to Prof. Peter Hore Comments

We thank Prof. Peter Hore for the careful reading of the manuscript and the high estimation of our results. Our response to the individual points of criticism is found below: while the reviewer's statements are printed in italics, our comments and changes are printed in standard font.

A nice manuscript. Publishable after minor corrections.
Scheme 1 appears on page 2 but is not referred to until page 7. Perhaps it should be moved nearer to the place where it's actually needed.

We have corrected this inaccuracy and referred Scheme 1 on page 2, when a study of Robert Kaptein and co-workers (Stob et al., 1989) is described.

Lines 35-36: it is an exaggeration to say that the radical-pair mechanism is "generally accepted" as the "explanation of bird navigation". Although it is clear that migratory birds have a magnetic compass sense, they also use the sun, stars, olfaction, landmarks etc. Additionally, the RPM hypothesis of magnetoreception is not yet "generally accepted".

This sentence has been rephrased as "A review on the radical-pair mechanism (RPM) of magnetoreception as a leading hypothesis to explain the bird navigation can be found in the literature (Hore and Mouritsen, 2016)".

Lines 41-42: "In a recently discovered DNA based magnetic sensor FAD was used to repair a DNA lesion by splitting a thymine dimer (Zwang et al., 2018)". This is a curious way to refer to DNA photolyase. I think the authors should at least say that $FADH^{-}$, the fully reduced form of FAD, acts as the chromophore and electron donor in this light-dependent DNA repair enzyme. It is also far from clear that there should be any magnetic field effect on this reaction (ACS Cent. Sci., 4 (2018) 318-320).

We have deleted this sentence for clarity.

I think the following two articles (at least) should be cited to avoid giving the impression that no-one other than Stob et al. (1989) has studied magnetic field effects on FAD photochemistry.

Murakami, M., K. Maeda, and T. Arai. 2005. Dynamics of intramolecular electron transfer reaction of FAD studied by magnetic field effects on transient absorption spectra. J. Phys. Chem. A 109:5793-5800.

Antill, L. M., and J. R. Woodward. 2018. Flavin adenine dinucleotide photochemistry is magnetic field sensitive at physiological pH. J. Phys. Chem. Lett. 9:2691-2696.

The articles mentioned above have been cited (Line 49).

Lines 63-64: I was puzzled by the mention of tryptophanyl radicals here. This only became clear in line 98. A few more words of explanation, or references to studies of FAD-Trp photo-reactions, would be helpful.

We extended the paragraph and wrote: Often FAD is discussed as a candidate molecule responsible for the formation of such spin-correlated radical pairs in living organisms that contain particular proteins - blue light photoreceptors, cryptochromes, which contain a non-covalently bound FAD photoreceptor molecule. The radical pair usually considered is a pair of radicals [$FAD^{\bullet-}$ $Trp^{\bullet+}$], which is formed by sequential electron transfer along the chain of tryptophan residues to the cofactor FAD in cryptochrome (Dodson et al., 2015). However, the appearance of the magnetic field effect in this secondary radical pair, [$FAD^{\bullet-}$ $Trp^{\bullet+}$], formed in parallel or subsequently from the FAD biradical might be significantly affected by the spin dynamics in the primary FAD biradical.

Line 190: it would be useful to have either a literature reference for this equation or some description of how it was derived.

We have added the following sentences describing derivation of the equation 1. "assuming a simple empirical model with two contributions to relaxation, one resulting from site-specific local field correlation time τ_c^i and another one being a field-independent constant. Accordingly, the total relaxation rate is given by the sum $R_1^{tot}(B) = \frac{R_1}{1+(\gamma_H B \tau_c)^2} + R_1^{inf}$". " Since the dominant relaxation mechanism of protons is the modulation of dipole-dipole coupling, the τ_c^i values are expected to be close to each over for a molecule with rigid structure due to overall molecular tumbling; the deviation of τ_c^i from average value highlights the molecular sites with increased or decreased mobility with respect to average. "

Lines 211-213: why does hydrogen peroxide cause the spectra to become sharper? Is this because it suppresses the comproportionation reaction, $FAD + FADH^- + H^+ \rightarrow 2FADH^{\bullet}$?

Yes, we think so.
We checked that the addition of H_2O_2 (with ~10 mM concentration) has prevented the photo-bleaching of the FAD sample like it was published in the work of Maeda et al. for FMN as a photosensitizer [Maeda, K., Lyon, C., Lopez, J., Cemazar, M., Dobson, C. & Hore, P.J. Improved photo-CIDNP methods for studying protein structure and folding. J. Biomol. NMR 16, 235–244 (2000)].

Lines 249-251: is the pH dependence of the CIDNP intensities evidence for a change in the stacking of the F and A groups?

R. Kaptein studied the dependence of photo-CIDNP and fluorescence of FAD on the pH in his work (Stob et al., 1989). These two dependences are astonishingly similar. A maximum of both CIDNP and fluorescence was observed at pH=2.4 with a sharp decrease at both low and high pH.

In our turn we have also checked the dependence of geminate CIDNP (CIDNP spectra were taken without delay after a short single laser pulse) of FAD on the pH of aqueous solution (see Fig. 1 below), and obtained curves that are very close to Kaptein's dependences.

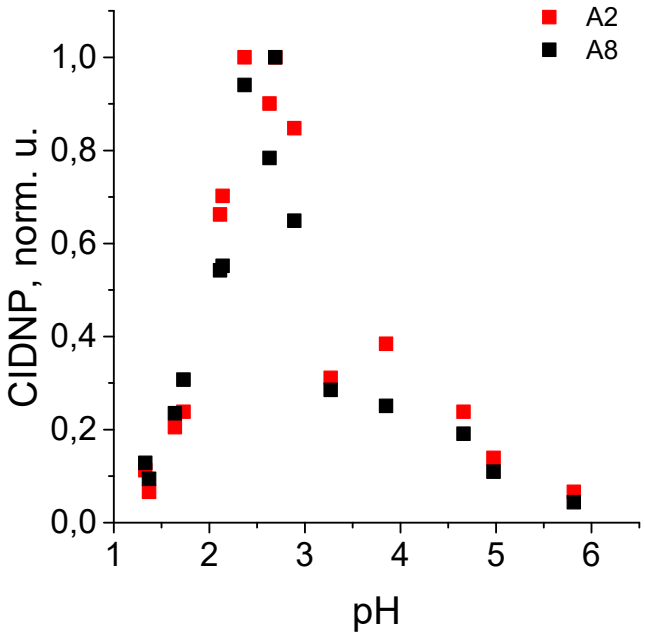

Fig.1. The pH-dependence of the geminate CIDNP intensity of adenine protons A2 (red) and A8 (black) of a 0.9 mM solution of FAD in D_2O; CIDNP spectra were taken without delay after a short single laser pulse by 64 scans; a 6 µs RF pulse was used for detection.

In Kaptein's work (Stob et al., 1989) there is the following reasoning why the CIDNP was observed only at narrow pH range: "the decrease of CIDNP in the high pH region is not due to a low yield of triplet flavins but is an inherent property of the biradical nature of the effect… In the low pH region the reduction of CIDNP intensity follows that of the fluorescence quenching…it is likely to be due to radiationless decay of the flavin excited singlets with concomitant reduction of the flavin triplet yield." "the fluorescence quenching at neutral pH in fact reflects the conformational equilibrium of ground-state FAD, since the lifetime of the singlet-state flavin is too short to allow for an equilibrium to be attained. We explain the pH-dependent CIDNP behavior also on the basis of a ground-state property of FAD, in this case the pKa of 3.6 of the adenine moiety. The reason is that now the protonation-deprotonation equilibria are slow compared to the CIDNP time scale of 10^{-7}-10^{-9} s (except for intramolecular proton transfer) so that the pH dependence is governed by the protonation state of the precursor molecule (FAD). The complete suppression of CIDNP at neutral pH indicates that the stacking equilibrium in the $FH^{\bullet}$-$A^{\bullet}$ biradical is shifted completely to the stacked form before T-S mixing can occur, in contrast to that of FAD itself where open conformations are present for about 20%."

Lines 272-274: "The high quality of the data left no doubts that only one maximum of CIDNP is detected in the field dependence excluding that two types of biradical with different exchange interaction are formed from FAD." What evidence do the authors have that there are not, in fact, two unresolved peaks? If there were, their results would not disagree quite so much with Stob, Kemminck and Kaptein.

We have made the following simulations suggested by P.J. Hore and referee #2, see Fig.4. We think it is clear from this figure that a second "open" conformation should give CIDNP at fields lower than 1 mT (at least in simulations), but we did not observe it in our experiments.

Here, numbers correspond to the following simulations:

1: normal distribution with its center at $r_0 = 0.89$ nm and a standard deviation $\sigma = 0.15$ nm - same as simulation 1 in main text;

2: normal distribution with $r_0 = 1.48$ nm and $\sigma = 0.15$ nm;

3: composite distribution made of equally weighted distributions used in sim.1 and sim.2;

4: composite distribution made of equally weighted distributions: a) $r_0 = 0.75$ nm $\sigma = 0.05$ nm, b) $r_0 = 1$ nm $\sigma = 0.05$ nm;

Apparently, the single maximum distribution used in sim.1 gives the best fit to the data.

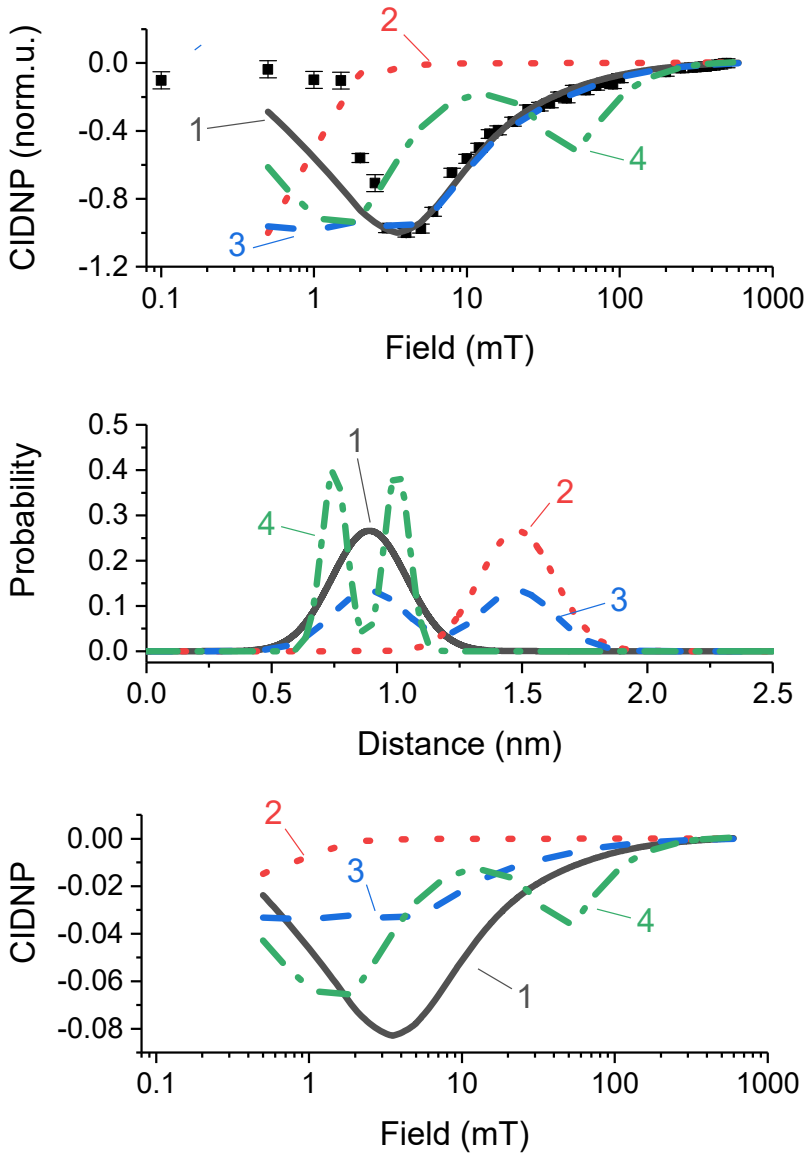

Fig. 4. Top – CIDNP field dependences: black squares – A8 experimental data, lines – simulation results for the model distributions 1-4 normalized to the maximum absolute experimental CIDNP intensity. Middle – distribution functions used in simulations 1-4. Bottom – calculated CIDNP field dependences for simulations 1-4 without normalization.

The simulations also presented in the answer to the Referee #2 comment (iv) show that models with two distinct biradical conformations lead to a pronounced deviation of the simulation results from the observed CIDNP field dependence. Although this does not mean that deviation of the biradical end-to-end distribution from a normal distribution is excluded, the merit of such deviation is anticipated to be relatively small. We believe that this allows us to talk about one type of the biradical conformation.

Table 1: should the units of G be s^{-2}?

Yes, it was a typo, there should be s^{-2}.

We hope that our manuscript in its present form is suitable for publication in the special issue of Magnetic Resonance dedicated to 80^{th} anniversary of Professor Kaptein.