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Physiology

Candidate genes mediating magnetoreception in rainbow trout (*Oncorhynchus mykiss*)

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Diverse animals use Earth's magnetic field in orientation and navigation, but little is known about the molecular mechanisms that underlie magnetoreception. Recent studies have focused on two possibilities: (i) magnetite-based receptors; and (ii) biochemical reactions involving radical pairs. We used RNA sequencing to examine gene expression in the brain of rainbow trout (*Oncorhynchus mykiss*) after exposure to a magnetic pulse known to disrupt magnetic orientation behaviour. We identified 181 differentially expressed genes, including increased expression of six copies of the *frim* gene, which encodes a subunit of the universal iron-binding and trafficking protein ferritin. Functions linked to the oxidative effects of free iron (e.g. oxidoreductase activity, transition metal ion binding, mitochondrial oxidative phosphorylation) were also affected. These results are consistent with the hypothesis that a magnetic pulse alters or damages magnetite-based receptors and/or other iron-containing structures, which are subsequently repaired or replaced through processes involving ferritin. Additionally, some genes that function in the development and repair of photoreceptive structures (e.g. *crggm3*, *purp*, *prl*, *gcip*, *crabp1* and *pax6*) were also differentially expressed, raising the possibility that a magnetic pulse might affect structures and processes unrelated to magnetite-based magnetoreceptors.

1. Introduction

Numerous animals detect Earth's magnetic field and use it to guide movements over a wide range of spatial scales [1]. Despite this, the molecular mechanisms underlying magnetoreception have remained enigmatic. Most recent studies have focused on two proposed mechanisms. The first is chemical magnetoreception, in which complex biochemical reactions are influenced by Earth's magnetic field [1,2]; the second involves crystals of the mineral magnetite, which interact with the ambient magnetic field and might transduce magnetic stimuli to the nervous system in several different ways (e.g. via hair cells or stretch receptors) [1,3,4].

One technique that has been used to distinguish between these mechanisms involves subjecting animals to a strong, brief magnetic pulse. This treatment is expected to have no lasting effect on chemical magnetoreception but might disrupt magnetite-mediated magnetoreception by altering the structure or pattern of magnetization in magnetite crystals [1,3,4]. Behavioural experiments have demonstrated that magnetic pulses do indeed alter or disrupt the magnetic orientation behaviour of several animals [1,3,5]. These findings are consistent with an effect on magnetite-based magnetoreception, although the possibility of a more general effect on the health or physiology of animals cannot be excluded with certainty [1].

Table 1. Summary of the RNA libraries generated for this study. Mapped reads passed quality criteria, aligned to the genome, and overlapped a protein-coding region. SRA, sequence read archive.

library	SRA accession	group	test date	raw reads ($\times 10^6$)	mapped reads ($\times 10^6$)
C9	SRR3623970	control	19 Aug 2015	27.0	10.0
C4	SRR3623965	control	26 Aug 2015	31.0	11.6
C10	SRR3623971	control	27 Aug 2015	28.0	10.2
C11	SRR3623958	control	28 Aug 2015	29.0	11.0
C12	SRR3623959	control	2 Sep 2015	27.9	10.3
P13	SRR3623960	pulsed	19 Aug 2015	26.4	9.6
P7	SRR3623968	pulsed	26 Aug 2015	30.3	11.1
P14	SRR3623961	pulsed	27 Aug 2015	30.4	11.1
P15	SRR3623962	pulsed	28 Aug 2015	28.9	10.8
P16	SRR3623963	pulsed	2 Sep 2015	28.5	9.7

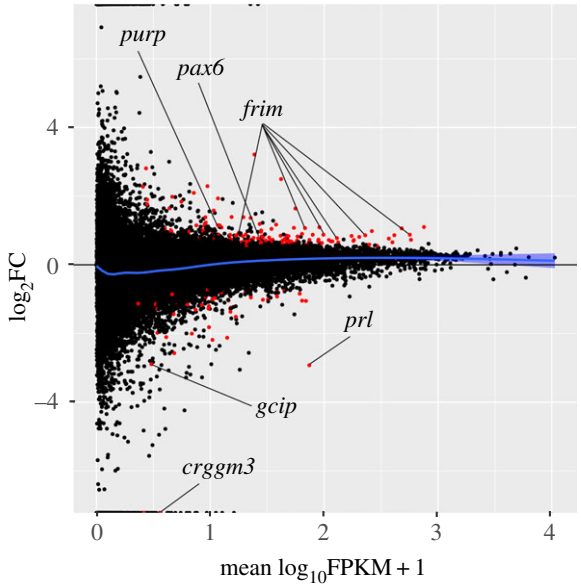

Figure 1. MA plot of the expression level ($\log_{10}$FPKM + 1) and ratio ($\log_2$FC) for each gene in pulsed relative to control trout. Genes with significantly different expression are shown in red. A smoothed function generated using a generalized additive model is provided (blue line). Genes mentioned in the text are labelled.

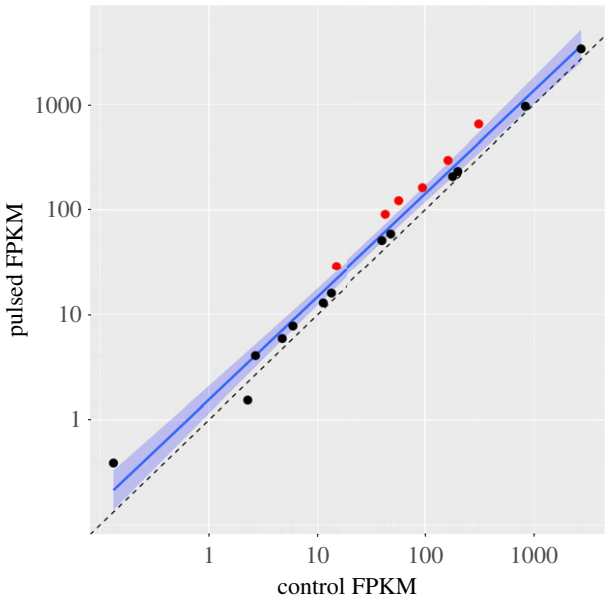

Figure 2. Expression of 19 annotated ferritin genes in the trout genome, including six copies of the gene *frim*, whose expression increased significantly (red points). The dashed line indicates equal expression between groups. The locally weighted scatterplot smoother (LOWESS, solid blue line) and its 95% CI (blue shaded region) are shown.

The rainbow trout *Oncorhynchus mykiss* is a promising species for studies of magnetoreception because trout respond to magnetic stimuli, candidate magnetoreceptor cells have been proposed and a draft genome sequence is available [6,7]. These factors make possible new approaches for investigating magnetoreception. Here, we report the first use of transcriptome sequencing to examine the effects of a pulsed magnetic field on gene expression, with a view towards identifying candidate genes that might function in the production or repair of magnetoreceptors.

2. Material and methods

Methods are summarized below; see the electronic supplementary material for a complete description of all procedures.

We randomly selected and exposed individual, captive-reared rainbow trout to either a 0.085 T magnetic field for 5 ms (pulsed group) or a sham exposure (control group) that included identical handling but lacked any pulsed magnetic field. Afterwards, fish were transferred to a new container, allowed to rest for 5 min, then euthanized. The brain was removed and stored at −80°C.

Total RNA was extracted from the brains of 20 individuals (10 control + 10 pulsed) and pooled in pairs for library preparation. RNA libraries (5 control + 5 pulsed) were barcoded, pooled and sequenced using two lanes of an Illumina HiSeq 2500 (125 bp, single-end reads). Raw sequence data were deposited into GenBank under accession number PRJNA324102 (table 1). All sequences were quality trimmed [8] and mapped [9] to the reference genome (GenBank accession CCAF010000000) [7]). Transcript abundance was quantified as the expected fragments per kilobase of exon per million mapped fragments (FPKM) and differences between groups were calculated as $\log_2$ of the expression ratio of pulsed relative to control groups ($\log_2$FC) [10].

We performed a de novo functional annotation of all proteins in the reference genome using [11]. Results were stored as gene ontology (GO) terms and functional enrichment of differentially

Table 2. Top 10 ranked GO terms from [11]. Each gene was scored and ranked by its ability to differentiate control and pulse-magnetized groups, and each term is then ordered by the average ranking (rank) of its associated genes. The number of genes assigned to each term (count) and their average score (score) are given. All GO terms are of three domains: biological process (BP), molecular function (MF) and cellular component (CC). The complete list of significant terms can be found in the electronic supplementary material.

GO term	average rank	average score ($\times 10^3$)	count	*p*-value	description	type
GO: 0016614	724.6	2.1	7	0.0001	oxidoreductase activity, acting on CH-OH group of donors	MF
GO: 0005007	724.6	2.1	7	0.0009	fibroblast growth factor-activated receptor activity	MF
GO: 0072669	748.6	1.5	6	0.0047	tRNA-splicing ligase complex	CC
GO: 0034464	757.3	1.2	8	0.0030	BBSome	CC
GO: 0003094	775.6	1.0	5	0.0291	glomerular filtration	BP
GO: 0021554	775.6	1.0	5	0.0295	optic nerve development	BP
GO: 0030517	775.6	1.0	5	0.0296	negative regulation of axon extension	BP
GO: 0005847	775.6	0.10	5	0.0304	mRNA cleavage and polyadenylation specificity factor complex	CC
GO: 0046914	779.8	1.1	9	0.0041	transition metal ion binding	MF
GO: 0038062	785	1.2	8	0.0043	protein tyrosine kinase collagen receptor activity	MF

expressed genes was assessed using a Fisher's exact test. Only functions with a false discovery rate (FDR) less than 0.05 were retained [11]. We used the non-parametric approach in [12] to identify GO features containing genes whose expression profiles can best cluster the groups of samples.

3. Results

We generated more than 285 million sequencing reads from 10 RNA libraries (electronic supplementary material, figure S1). On average, 10.5 (s.d. 0.67) million reads per library passed quality control and mapped to annotated genes in the reference genome (table 1). Of the 46 585 annotated genes, 38 550 (83%) contained at least 10 overlapping reads across all libraries and were assessed for expression level (electronic supplementary material, figure S2). We identified 181 genes differentially expressed in response to the pulsed magnetic field (figure 1; electronic supplementary material, figure S3). These genes were enriched for 15 GO annotations (electronic supplementary material, table S1), most specifically ferric iron binding (FDR $= 2 \times 10^{-6}$), iron ion transport (FDR $= 3 \times 10^{-5}$) and cellular iron ion homeostasis (FDR $= 3 \times 10^{-5}$). Eighteen (95%) of 19 ferritin-coding genes in the trout genome increased expression, including significant increases in six copies of the gene *frim*, which encodes the middle subunit of the ferritin protein (figure 2). Of the 6899 GO annotations assigned to the genome, 69 (1%) were significantly linked to differences between control and pulsed groups (table 2; electronic supplementary material, table S2). The highest-ranking terms were the functions of oxidoreductase activity ($p = 0.0005$) and fibroblast growth factor-activated receptor activity ($p = 0.0009$).

4. Discussion

(a) Iron regulation and magnetoreception

If the receptors for the magnetic sense are based on magnetite, then they may be disrupted by a magnetic pulse [1,3–5]. Results revealed increased expression of ferritin in fish exposed to a magnetic pulse. Ferritin is a polymeric protein that acts as a repository to store excess iron within cells [13]. Inside ferritin, up to 4500 iron atoms are oxidized and stored as hydrated iron oxides, including superparamagnetic, ferromagnetic and ferrimagnetic crystals [13,14]. It is possible that the magnetic pulse resulted in the disruption or liberation of iron oxide crystals from ferritin [4] or other iron-containing structures (e.g. MagR, MagR/Cry) [15]. If so, then excess free iron might account not only for the increase in ferritin expression (for the purpose of sequestration), but also for the differences in activities associated with the oxidative consequences of free iron. The latter include oxidoreductase activity, transition metal ion binding, electron transport chain complexes and DNA damage repair. Previous studies have implicated ferritin in the biomineralization of magnetite [16,17]. Thus, ferritin might be involved in producing or repairing magnetite-based magnetoreceptors after a magnetic pulse.

(b) Genes associated with photosensitive structures

Several genes that function in the development, maintenance or repair of photosensitive structures and pathways (*crggm3*, *prl*, *purp*, *crabp1 pax6* and *gcip*) were differentially expressed. Meanwhile, no significant differences in the expression of cryptochromes, photosensitive proteins hypothesized to function in chemical magnetoreception [2], were observed. Among the genes affected were some implicated in development of the optic nerve and habenula, two neural structures that link photoreceptive organs (retinae and pineal gland, respectively) to the brain. Both the retina and pineal gland have been considered possible locations of magnetoreception [1–3,18,19], although only the pineal gland, including non-visual encephalic photoreceptors, was included in the brain tissue sampled in this study.

Both *purp* and *crabp1* bind and transport vitamin A derivatives between and within, respectively, cells of the retinal pigment epithelium and interphotoreceptor matrix [20].

These vitamin A derivatives are eventually metabolized into 11-*cis*-retinal, the chromophore required for vision. Also known as retinol binding protein 4, *purp* acts as a neurite-sprouting factor and is important for optic nerve regeneration [21]. One of the largest reductions in expression was in the hormone prolactin (*prl*). Prolactin has been shown to stimulate the synthesis of visual pigments in the retinae of rainbow trout [22]. However, this hormone has a broad range of functions, thus making its role in responding to the magnetic pulse unclear.

Why a magnetic pulse altered expression of genes related to photosensitive structures is not known. One possibility is that magnetic particles are closely associated with these structures so that pulse-induced movement of the particles damaged surrounding tissue and elevated gene expression needed to repair this 'collateral damage'. Alternatively or additionally, the pulse might have generated magnetophosphenes by activating photosensitive structures, or otherwise exerted an effect through unknown mechanisms.

5. Conclusion

Our study is the first to use a transcriptomic approach to investigate the effect of magnetic pulses on gene expression, with a view towards identifying candidate genes involved in magnetoreception. Results demonstrate that expression of ferritin genes is elevated after a pulse, a finding consistent with the hypothesis that ferritin is involved in generating or repairing magnetite-based magnetoreceptors. In addition, the magnetic pulse altered expression of genes implicated in the development, function and repair of visual elements. Additional studies examining the response of candidate genes (e.g. using quantitative polymerase chain reaction, qPCR) [23] are needed to extend the work to other relevant tissues (e.g. retina, pineal gland and habenula) and to other magnetically sensitive species, as well as to elucidate the functional significance of the patterns of gene expression observed.

Ethics. Experimental methods were approved by Duke University's IACUC (protocol A175-15-06).

Data accessibility. Raw sequence data were deposited into GenBank under accession PRJNA324102 (also see table 1). All mapping, expression and annotation data are available in Dryad (http://dx.doi.org/10.5061/dryad.73b86) [24].

Authors' contributions. R.R.F. designed the study, collected and analysed data, and drafted the manuscript. B.R.W. collected data and helped draft the manuscript. D.A.E. and K.J.L. helped design the study, collect data and draft the manuscript. S.J. conceived, designed and coordinated the study and helped draft the manuscript. All authors gave final approval for publication and agree to be held accountable for the content herein.

Competing interests. We declare we have no competing interests.

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