Sperm head abnormalities are associated with excessive omega-6 fatty acids in two finch species feeding on sunflower seeds

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Abstract

Sunflower seeds are commonly provided in garden bird feeders, but contain high levels of linoleic acid (LA), an omega-6 (ω-6) polyunsaturated fatty acid (PUFA). LA is associated with increased oxidative stress, which can damage cell membranes, and in particular sperm cells. We investigated the relationship between the proportion of LA in the blood (as well as the proportion of 10 other fatty acids, the proportion of total ω-6 and ω-3, and ω-6/ω-3 ratio) and sperm quality (the frequency of sperm head abnormalities and sperm swimming speed) in two seed-eating finch species, greenfinches *Chloris chloris* and hawfinches *Coccothraustes coccothraustes*. The majority of birds were sampled in urban environments with direct access to sunflower seeds, whereas a smaller group were sampled in a rural area. We found that both species, but particularly greenfinches caught near feeders, exhibited levels of LA that were considerably higher than those reported for other wild birds in the literature, and higher than other bird species sampled at the same site. Furthermore, we found that the proportion of LA was positively correlated with the frequency of abnormal sperm heads (sperm missing the acrosome), while there was no significant effect of fatty acid composition on sperm swimming speed. Our results indicate that the sperm quality of finches may be negatively affected by a high intake of sunflower seeds, adding to a growing body of research showing that supplementary feeding can have detrimental side effects for urban animals. This is particularly relevant for the greenfinch, which is currently affected by disease and population declines.

Introduction

Bird feeders are a common feature of the urban environment, and have become increasingly important as a food source for urban birds. In the western world, it is estimated that around half of households provide food for wild birds in their gardens (Jones and Reynolds 2008; Davies et al. 2009), also during the breeding season (Orros & Fellowes 2015). Such supplementary feeding may enhance bird survival and reproductive success (Robb et al. 2008; Fuller et al. 2012); however, there has been concern about potential negative effects, such as increased incidence of disease (Robb et al. 2008; Robinson et al. 2010). In addition to the potential disease transmission risks of aggregated birds at feeders, recent research shows that the nutritional composition of provided foods can negatively influence body condition and reproductive success of wild birds (Plummer et al. 2018). Households tend to provide foods that would not be found abundantly in the local environment, for example bread, peanuts, or sunflower seeds (Jones and Reynolds 2008; Støstad et al. 2017), and birds may not have evolved the capability to judge the nutritional value of such novel food items. Although urban birds select the most energy-rich human-based food types when offered a choice (Støstad et al. 2017), such foods are often high in calories yet inadequate in other vital aspects, e.g. vitamin content or fatty acid composition, and indiscriminate consumption could lead to malnutrition.

Polyunsaturated fatty acids (PUFAs) are fatty acids that are crucial for a number of important processes such as immune function and brain development (Patterson et al. 2012), but can also be detrimental when consumed in excess. In particular, a key dietary challenge is achieving a balanced ratio between omega-3 (ω-3) and omega-6 (ω-6) PUFAs. Excessive amounts of ω-6 PUFAs can lead to oxidative damage and increased inflammatory responses in both birds and humans (e.g. Larsson et al. 2004; Cherian 2007; Brown 2016). For example, linoleic acid (LA, 18:2n-6), one of the most abundant ω-6 PUFAs in terrestrial plants (Hixson et al. 2015) and thus an important component in the diet of many animals, can be metabolised into reactive oxygen species such as hydrogen peroxide (Brown 2016). When such reactive oxygen species are not balanced by sufficient amounts of antioxidant enzymes, they cause oxidative stress, which damages cell membranes through lipid peroxidation. On the other hand, whilst excessive ω-6 PUFAs can be detrimental, it is also important to ensure adequate intake of ω-3 PUFAs. These ω-3 PUFAs are essential for cell membrane structure and flexibility, and deficiency can cause poor growth, impaired vision and a number of other detrimental effects (Brenna and Carlson 2014; Twining et al. 2016a). Indeed, a high intake of dietary ω-3 PUFAs has been shown to improve chick fitness in tree swallows (Twining et al. 2016b). Importantly, ω-3 and ω-6 PUFAs compete for digestive enzymes, which means that a higher intake of ω-3 can decrease the amount of ω-6 that is metabolised into reactive oxygen species (Brown 2016), emphasising the need for a balance between the two.

Notably, many food types provided to wild birds by households are rich in ω-6 PUFAs. In particular, sunflower seeds, which are commonly used in bird feeders, have a PUFA profile consisting almost exclusively of LA with very low levels of ω-3 PUFAs (see Table 1). Thus, urban birds that use bird feeders extensively may have an unbalanced diet. Isaksson et al. (2017) found that urban house sparrows *Passer domesticus* had higher plasma levels of LA than rural birds, possibly due to access to different food sources, although they found the opposite pattern for great tits, indicating species differences in either diet or metabolism. Sparrows are predominantly seed eaters, as opposed to the more omnivorous great tits, so that sparrows may be more affected by varying availability of LA-rich seeds. In Norway, providing sunflower seeds for wild birds has become increasingly popular during the last few decades, as demonstrated by a gradual increase in the annual import of sunflower seeds intended for animal feeding from zero in 1993 to nearly 8 million kg in 2017, with an annual import value of 6 million USD in 2017 (Statistics Norway, 2018). Thus, investigating the physiological effects of feeding sunflower seeds to wild birds is highly relevant.

Avian sperm membranes are largely composed of ω-6 PUFAs (Surai et al. 1998; Cerolini et al. 2003), so ω-6 PUFAs are in fact essential to avian spermatogenesis for production of sperm membranes. However, ω-6 PUFAs are at the same time potentially harmful to sperm quality through the production of reactive oxygen species as described above. The unsaturated fatty acids in sperm membranes are unstable and bond readily with reactive oxygen species (Gagnon et al. 1991), which oxidises the lipid membranes of the sperm, ultimately causing disruption of sperm functions (Aitken and Baker 2006, Bonisoli-Alquati et al. 2011). It is important that the membrane of the acrosome (the anterior part of the sperm head) remains intact until the point of fertilisation, as the enzymes contained within the acrosome are necessary for the acrosome reaction, allowing sperm to penetrate the ovum (Nishio and Matsuda 2017). The apparent contradiction between the beneficial and detrimental impacts of ω-6 PUFAs means that a balanced intake of ω-6 is essential to ensure optimal sperm quality in birds. Moreover, sperm quality may be particularly important for male fitness in many passerine bird species due to the high incidence of promiscuity and subsequent sperm competition (Griffith et al. 2002; Pizzari and Parker 2009), so that deficiencies or overconsumption of ω-6 PUFAs could have considerable fitness consequences for these birds.

In captive bird populations, the effect of dietary PUFA content on sperm performance appears to be variable, and depends on age and levels of other nutrients such as vitamin E (Cerolini et al. 2003; Khatibjoo et al. 2018). Diets enriched with ω-3 PUFAs have been associated with increased fertility (Blesbois et al. 1997; Feng et al. 2015) and larger semen volume (Surai et al. 2000) compared to ω-6 rich diets; although one study showed that an ω-3-rich diet led to a slight decrease in sperm motility yet an increase in fertility (Kelso et al. 1997), and other studies have found no

Table 1. Nutritional content of sunflower seeds. Data from USDA National Nutrient Database for Standard Reference, https://ndb.nal.usda.gov/ndb/. Note that the retailer of the provided sunflower seeds was unable to provide the exact nutrient content; additionally, nutritional content may vary slightly among sunflower varieties and batches. C:D = number of carbon atoms:double bonds. SFA = saturated fatty acids, MUFA = mono-unsaturated fatty acids, PUFA = poly-unsaturated fatty acids.

Trivial name (abbreviation)	C:D	Class	g (of 100 g)
Palmitic acid	C16:0	SFA	2.21
Stearic acid	C18:0	SFA	1.69
Oleic acid	C18:1 n-9	MUFA	18.38
Linoleic acid (LA)	C18:2 n-6	ω-6 PUFA	23.05
Gamma-linolenic acid (GLA)	C18:3 n-6	ω-6 PUFA	0
Dihomo-gamma-linolenic acid (DGLA)	C20:3 n-6	ω-6 PUFA	0
Arachidonic acid (ARA)	C20:4 n-6	ω-6 PUFA	0
Alpha-linolenic acid (ALA)	C18:3 n-3	ω-3 PUFA	0.06
Eicosapentaenoic acid (EPA)	C20:5 n-3	ω-3 PUFA	0.01
Docosapentaenoic acid (DPA)	C22:5 n-3	ω-3 PUFA	0
Docosahexaenoic acid (DHA)	C22:6 n-3	ω-3 PUFA	0
Total ω-6 PUFA			23.05
Total ω-3 PUFA			0.07
Ratio ω-6 / ω-3			329.29

difference in sperm motility (Cerolini et al. 2003; Khatibjoo et al. 2018). In comparison, research on fatty acid composition in the sperm of wild birds is scarce, and to our knowledge there are no studies on the impact of the nutritional content of the diets of wild birds on sperm quality. However, oxidative stress is negatively associated with sperm motility and swimming speed in wild great tits *Parus major* (Helfenstein et al. 2010) and barn swallows *Hirundo rustica* (Bonisoli-Alquati et al. 2011), and since an unbalanced fatty acid composition in the diet can aggravate oxidative stress, a diet rich in ω-6 PUFAs could potentially be detrimental for sperm quality in wild birds.

Here, we first quantify the fatty acid composition in the blood of two seed-eating finch species, the greenfinch *Chloris chloris* and the hawfinch *Coccothraustes coccothraustes,* caught at sunflower seed feeders, and we compare these results to the fatty acid composition of a smaller number of individuals of six other common garden bird species. Secondly, we investigate the relationship between sperm performance and the proportion of LA in the blood of the two finch species; using birds that were caught in proximity to sunflower feeders as well as birds that were caught in rural, feeder-poor areas. We predict that seed-eating finches, especially those caught in proximity to feeders, will have higher blood levels of LA than other species, and that individuals with higher levels of LA will have lower sperm quality (as expressed by the proportion of abnormal sperm heads and sperm swimming speed).

Methods

Study species

The two focal species of the study, greenfinches and hawfinches, were chosen mainly due to their feeding ecology as seed eaters with a preference for sunflower seeds. Personal observations as well as the literature (e.g. Brittingham and Temple 1992) suggest that both species, but particularly

Table 2. Sperm head abnormalities in greenfinches across four years of sampling. Samples from 2012 and 2013 are not included in further analyses.

Year	Number of samples	Percent abnormalities	Standard deviation
2012	6	4.17	4.36
2013	13	35.23	22.95
2015	46	7.58	8.03
2017	22	12.36	21.00

greenfinches, are frequent visitors to sunflower feeders, and that they tend to include sunflowers as a substantial part of their diet (as opposed to e.g Parids (tits), which will also frequently visit feeders with other food types e.g. fats). Moreover, greenfinch populations are declining in the UK (Harris et al. 2018), in Norway (Falkenberg et al. 2018), and in Czechia (Reif et al. 2010), and the decline in the UK has been linked to disease spreading through their use of feeders (Robinson et al. 2010). As such, investigating further adverse effects from supplementary feeding on this species is particularly important, as it could help inform potential conservation actions. Finally, we have in the past observed remarkably high rates of abnormal sperm in the greenfinch (Table 2), especially in 2013 when several samples showed around 50% sperm abnormalities (unfortunately we do not have fatty acid profiles for these older samples).

Sampling

For the current study, we sampled birds in two countries (Norway and Czechia) and across two years (2015 and 2017). We had variable catching success and there was variability in the quality of the collected data, i.e. some males for which we obtained fatty acid data had no sperm, and some had sufficient sperm for morphology assessment but not for sperm velocity assessment. We have aimed to maximise sample sizes in each analysis, rather than limiting analyses to the individuals for which we had all types of data; this means that sample sizes vary among the analyses.

The main part of the sampling was done in southeast Norway, in urban or suburban areas (see list of locations in Table S1), during the breeding season (April – June) of 2015 and 2017. At these sampling sites, we provided sunflower seeds in feeders throughout the preceding winter and up until (and including) the period of sampling. Sunflower seeds were from the Norwegian retailer Felleskjøpet, see nutrient content in Table 1. The purpose of the feeding was to maximise the chances of catching birds that had utilised sunflower feeders; however, we do not know to what extent individual birds used the feeders, and additionally, there is no guarantee that the sampled birds were overwintering residents at the sampling site. Still, our observations indicate that finches at

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the sampling sites used the feeders frequently and for long periods of time, so we consider it likely that sunflower seeds constitute a considerable part of the diet of the sampled birds.

1   At the feeder sites, we caught 63 greenfinches and 11 hawfinches across the two sampling
2   years (Table S1). To compare the fatty acid profiles of these individuals with other local species, in
3   particular species that tend to forage less at the sunflower feeders (based on personal observation
4   and general species ecology), we sampled a small number of birds of other species from the feeder
5   sites. These were eight siskins *Spinus spinus*, six chaffinches *Fringilla coelebs*, two great tits *Parus*
6   *major*, two blue tits *Cyanistes careuleus*, two coal tits *Periparus ater*, and one goldfinch *Carduelis*
7   *carduelis*. These were not used for sperm analysis. Additionally, three greenfinches and eight
8   hawfinches were caught in rural areas in southern Czechia in 2015, with limited access to feeders
9   (Tryjanowski et al. 2015). Sampling in an area with a low density of feeders as well as the areas with
10   direct access to well-stocked feeders allowed us to catch birds that were likely to have ingested
11   variable amounts of sunflower seeds, facilitating a correlational analysis between LA and sperm
12   performance.

13   Birds were caught using mist nets. Blood samples were collected from the brachial vein, and
14   a drop of blood was deposited on a blotting paper (Whatman 903 Protein Saver Card, Sigma-Aldrich)
15   for subsequent fatty acid analyses. Sperm samples were taken using cloacal massage (Wolfson 1952;
16   Kucera and Heidinger 2018), and upon collection all samples were fixed and stored in 5%
17   formaldehyde solution buffered with PBS. For a subset of samples (all greenfinches from 2015 from
18   Norway) sperm were also used for velocity analysis, see below. All sampling was done in adherence
19   with local guidelines for animal research and with all relevant permits. Birds were released
20   immediately after sampling.

21   **Sperm morphology assessment**
22   Seven greenfinches (all from the Norwegian sites) had insufficient sperm and were used only for fatty
23   acid analysis, and so in total 59 greenfinches and 19 hawfinches from both countries were used for
24   sperm morphology assessment. To measure the proportion of morphologically abnormal sperm in a
25   sample, we prepared sperm smears of the formaldehyde-fixed sperm. Sperm solution was smeared
26   on a glass slide, air-dried, and rinsed with distilled water, before being assessed using a digital light
27   microscope (DM6000 B, Leica Microsystems) set to 320× magnification. Images were taken with a
28   digital camera (DFC420, Leica Microsystems, Heerbrugg, Switzerland) mounted on the microscope.

29   We aimed to assess at least 100 randomly selected sperm from each male, scoring each cell
30   as having either 1) a morphologically normal sperm head, defined as a head with no obvious signs of
31   damage and a shape that corresponded to the usual appearance for the species (fig. S1a); 2) a sperm

6

32 that was missing the acrosome (fig. S1b); or 3) a misshapen sperm head that retained the acrosome

33 but appeared abnormal in shape, for example had lost its helical appearance or was shaped like a

34 hook (fig. S1c). In this study, we place slightly more emphasis on the category of missing acrosomes,

35 as this is a well-defined sperm defect which is likely related to a premature acrosome reaction,

36 whereas 'misshapen sperm heads' is a more vaguely defined trait and was also more difficult to

37 assess accurately. All samples were assessed by the same person (HNS) who had no further

38 information on the sample at the time of assessment.

39 **Sperm velocity assessment**

40 Sperm samples were collected as described above and immediately diluted in Phosphate Buffered

41 Saline (PBS) pre-heated to 40°C. Next, 6 μl of the sperm suspension was loaded into a chamber slide

42 (depth 20 μm, Leja®, Netherlands) and sperm motion was recorded for six unique fields of view (5s

43 each for a total of 30s) at 400x using phase contrast microscopy (CX41, Olympus, Japan) connected to

44 a digital camera (Legria HF S200, Canon, Japan). All samples, chamber slides, and the heated

45 microscope stage (TP-S, Tokai Hit, Shizuoka, Japan) were maintained at a constant temperature of

46 40°C throughout.

47   Videos of sperm motion were analysed using computer-assisted sperm analysis (CASA; HTR-

48 CEROS sperm tracker, CEROS v.12, Hamilton Thorne Research) with frame rate set at 50 Hz and 25

49 frames (i.e. sperm cells were tracked for 0.5 s) and quality control settings following Rowe et al.

50 (2015). Each analysis was visually examined and cell detection parameters established to exclude

51 non-sperm, contaminating particles: minimum contrast, 50-100; minimum cell size, 8-12 pixels;

52 sperm head elongation (i.e. width/length), < 75%. Additionally, we excluded non-continuous sperm

53 tracks or sperm tracked for less than 10 frames, as well as tracks for which the maximum frame-to-

54 frame movement exceeded the average frame-to-frame movement by 4 SDs for the same track, as

55 such tracks tend to represent tracking errors. Finally, to account for the effects of drift, tracks with an

56 average path velocity (VAP) of <35 μm s^{-1} or a straight line velocity (VSL) of <25 μm s^{-1} were

57 considered immotile. These cut-offs were based on an analysis (unpublished data) of dead sperm

58 cells using the same recording and analysis conditions as those used in the current study. All CASA

59 was done by the same person (MR) and without information about the male's identity.

60   Following this filtering process, we excluded samples with less than 5 motile sperm tracks

61 from analyses of sperm swimming speed. After this exclusion, the total number of motile sperm

62 tracked for each male ranged from 5 to 60 (median = 27, mean = 29.08 ± 19.28), and the total

63 number of samples with swimming speed data was 24. For each sperm cell, we recorded cell straight-

64 line velocity (VSL, i.e. average velocity on a straight line between the start and endpoint of the sperm

65 track), average path velocity (VAP, i.e. average velocity over a smoothed sperm track), and curvilinear

66 velocity (VCL, i.e. velocity over the actual sperm track). VSL, VAP and VCL were all strongly correlated

67 (Pearson's correlation, all r > 0.80), and so we used VCL as an indication of sperm swimming speed.

68 **Fatty acid extraction and gas chromatography analysis**

69 Fatty acid analysis of blood samples was performed by Vitas AS (vitas.no), Norway. One punch of

70 whole blood (6 mm diameter of blotting paper) was diluted with 900 µL 0.5M sodium methylate.

71 After methylation (20 min, 450rpm, 50°C), fatty acid methyl esters (FAMEs) was extracted with 500

72 µL hexane and 300 µL distilled water. After thorough mixing (5 min) and centrifugation (5 min, 4000

73 g), 3 µl of the aliquot was injected into a Gas Chromatography - Flame Ionization Detector (GC-FID).

74 GC-FID was performed with an Agilent 7890A Gas Chromatograph System (Agilent Technologies, Palo

75 Alto, CA, USA). The fatty acids were separated on a Supelco SP2380 (30m x 250µm x 0.2µm) column.

76 The results are reported as area-% of FAMEs.

77 **Statistical analysis**

78 All statistical analysis was done in R v 3.3.3 (R Core Team 2017). To analyse the relationship between

79 the proportion of LA and sperm head abnormalities, we constructed two generalised linear mixed

80 models (GLMM) using the glmmTMB package (Brooks et al. 2017), with the proportion of LA in the

81 blood as a predictor variable, and either 1) the proportion of sperm with missing acrosomes or 2) the

82 proportion of misshapen sperm heads as response variables. To account for the effects of species

83 and sampling, we created a single compound variable which signified the country of collection, year

84 of collection, and species (e.g. NO17GF), which was used as a random effect in all models. Note that

85 using other combinations of random effects (e.g. only including 'year' and 'species', or using a nested

86 random effect structure with year/country/species), produced similar results (not shown). Due to the

87 non-normality of the sperm abnormality data (strong left skew, but not zero-inflated), we used a

88 negative binomial distribution with a log link function, which provided better model fit than

89 alternative model parameters. This was assessed using the plot(model) function in R (Q-Q plots and

90 similar) as well as with the DHARMa package (Hartig 2017). The glmmTMB models also corrected for

91 overdispersion.

92 Although the relationship between LA and sperm abnormalities was the main interest in the

93 current study, we also present the relationship between 10 other measured fatty acids and sperm

94 abnormalities, using similar GLMM models. The proportion of each fatty acid was the predictor

95 variable in each model, with either the proportion of sperm with a missing acrosome or the

96 proportion of misshapen sperm heads as a response variable. Finally we constructed three further

97 GLMM models using 1) the total proportion of ω-6 PUFAs, 2) the total proportion of ω-3 PUFAs, and

98 3) the ω-6/ω-3 ratio as the predictor variables. These additional models were otherwise the same as

99 the models with LA.

100 Three individuals (all hawfinches from Norway) showed particularly high levels of misshapen

101 sperm heads (outside 4 times the interquartile range), and we suspect this was due to some

102 unrelated factor such as disease. When these outliers were included in the models, this invalidated

103 model assumptions based on visual analysis of the model plots. We therefore removed these

104 individuals from the analyses. Results from models including the outliers are presented in Table S2.

105 To analyse the relationship between the proportion of LA and sperm swimming speed (in the

106 subset of greenfinches from Norway from 2015), we used a linear mixed effect model (LME), with

107 VCL for each motile sperm cell as a response variable, the individual proportion of LA as a predictor

108 variable, and male ID as a random effect. We also constructed 13 similar LME models using the 10

109 other fatty acids, total ω-6 PUFAs, total ω-3 PUFAs, and the ω-6/ω-3 ratio as predictors.

Results

111 Linoleic acid (LA, 18:2n-6) was the most abundant fatty acid in the blood of both greenfinches and

112 hawfinches, as it accounted for 36.2% of all fatty acids in the greenfinch (Fig. 1a) and 28.3% in the

113 hawfinch (Fig. 1b). The proportion of LA was higher in these two species compared to other sampled

114 species (Fig. 2), although the chaffinches also exhibited relatively high proportions of LA. Nearly half

115 of total fatty acids in the greenfinch were ω-6 PUFAs (46.2%), versus 33.5% in the hawfinch. In

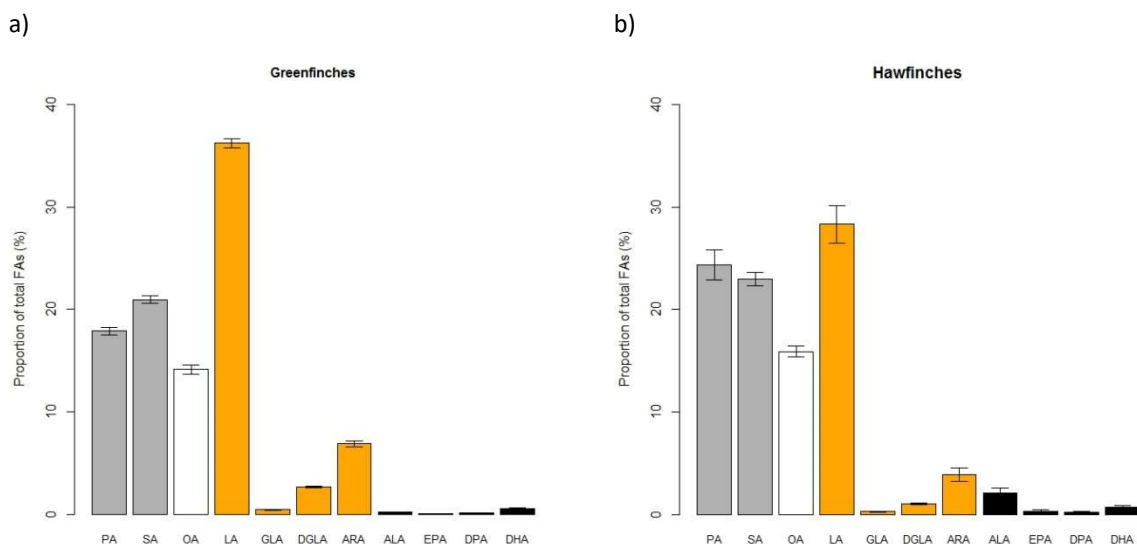a)   b)

Figure 1. Proportion of fatty acids (% of total fatty acid content) in a) greenfinches (n = 66) and b) hawfinches (n = 19). Light grey bars are saturated fatty acids (SFA), white bars are mono-unsaturated fatty acids (MFAs), orange bars are ω-6 poly-unsaturated fatty acids (PUFAs), and black bars are ω-3 PUFAs. Error bars are +/- 1 standard error. PA = Palmitic acid, SA = Stearic acid, OA = Oleic acid, LA = Linoleic acid, GLA = Gamma-linolenic acid, DGLA = Dihomo-gamma-linolenic acid, ARA = Arachidonic acid, ALA = Alpha-linolenic acid, EPA = Eicosapentaenoic acid, DPA = Docosapentaenoic acid, DHA = Docosahexaenoic acid.

contrast, ω-3 PUFAs accounted for only 0.9% of total FAs in the greenfinch and 3.3% in the hawfinch. The mean ω-6/ω-3 ratio (the mean of all individual ω-6/ω-3 ratios) was 75.7 for the greenfinch and 29.4 for the hawfinch.

The proportion of LA was lower in birds caught in a feeder-poor area in Czechia than in birds caught in proximity to sunflower feeders in Norway (23.6% versus 36.3% respectively, Fig. 3), and the total proportion of ω-6 PUFAs was also lower in the Czech birds (27.7% versus 46.1%). Conversely, the total proportion of ω-3 PUFAs was higher in Czech birds than in Norwegian birds (4.1% versus 0.9%). For all mean fatty acid values, see table S3 (all greenfinches and hawfinches used for analysis) and S4 (all eight species sampled in Norway in 2015).

The percentage of sperm with a missing acrosome varied from 0% to 32% (mean: 6.1%) in individual males, with higher mean rates in greenfinch than in hawfinch males (6.5% versus 4.8% respectively), and lower rates in 2015 than in 2017 (5.7% versus 7.4%). The proportion of sperm with a missing acrosome was positively correlated with the proportion of LA in the blood (Table 3, Fig. 4) and with the total proportion of ω-6 PUFAs (Table 3). Additionally, there was a tendency towards a negative correlation with eicosapentaenoic acid (EPA, $p = 0.067$, Table 3). Notably, all correlations between sperm with a missing acrosome and ω-6 PUFAs were positive, whereas all correlations with ω-3 PUFAs were negative.

The proportion of sperm with a misshapen sperm head varied from 0% to 15% (excluding outliers; range incl. outliers 0% - 72%). The proportion of sperm with misshapen heads was not related to the proportion of LA in the blood (Table 4), while there was a positive correlation between sperm with misshapen heads and gamma-linolenic acid (GLA, Table 4).
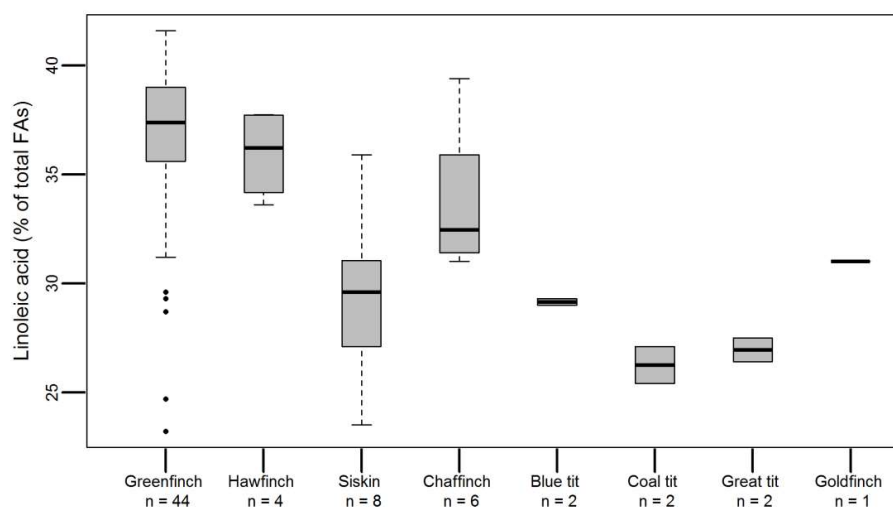

Figure 2. The level of linoleic acid (C18:2n-6, % of total fatty acids) in the blood of eight bird species caught near sunflower feeders in Norway in 2015.

There was no relationship between LA and sperm swimming speed in the 24 greenfinches for which we had sperm swimming speed data, although the trend was negative (t = -1.36, p = 0.19, *r* = -0.28 (-0.58, 0.14)). Similarly, there was no correlation between sperm swimming speed and any other aspect of fatty acid composition (Table S5).

Table 3. Generalised linear mixed models showing the relationship between the proportion of fatty acids in the blood and the proportion of sperm with a missing acrosome, in 59 greenfinches and 19 hawfinches. A single variable indicating the year, species and the country of collection was included as a random effect in all models. C:D = number of carbon atoms:double bonds, LCL = lower confidence limit, UCL = upper confidence limit. Significant results are presented in bold.

Predictors	C:D	Response	df	z	p	r	LCL, UCL
Palmitic acid	C16:0	Sperm with no acrosome	74	-1.616	0.106	-0.18	-0.38, 0.04
Stearic acid	C18:0	Sperm with no acrosome	74	-1.625	0.104	-0.19	-0.39, 0.04
Oleic acid	C18:1 n-9	Sperm with no acrosome	74	-1.127	0.260	-0.13	-0.34, 0.10
Linoleic acid (LA)	**C18:2 n-6**	**Sperm with no acrosome**	**74**	**2.491**	**0.013**	**0.28**	**0.06, 0.46**
Gamma-linolenic acid (GLA)	C18:3 n-6	Sperm with no acrosome	74	0.211	0.833	0.02	-0.20, 0.24
Dihomo-gamma-linolenic acid (DGLA)	C20:3 n-6	Sperm with no acrosome	74	0.625	0.532	0.07	-0.15, 0.29
Arachidonic acid (ARA)	C20:4 n-6	Sperm with no acrosome	74	1.433	0.152	0.16	-0.06, 0.37
Alpha-linolenic acid (ALA)	C18:3 n-3	Sperm with no acrosome	74	-1.615	0.106	-0.18	-0.38, 0.04
Eicosapentaenoic acid (EPA)	C20:5 n-3	Sperm with no acrosome	74	-1.833	0.067	-0.21	-0.40, 0.02
Docosapentaenoic acid (DPA)	C22:5 n-3	Sperm with no acrosome	74	-1.433	0.152	-0.16	-0.37, 0.06
Docosahexaenoic acid (DHA)	C22:6 n-3	Sperm with no acrosome	74	-0.588	0.557	-0.07	-0.28, 0.16
Total ω-6 PUFA		**Sperm with no acrosome**	**74**	**2.275**	**0.023**	**0.26**	**0.03, 0.44**
Total ω-3 PUFA		Sperm with no acrosome	74	-1.719	0.086	-0.20	-0.40, 0.03
Ratio ω-6 / ω-3		Sperm with no acrosome	74	0.532	0.594	0.06	-0.16, 0.28

Table 4. Generalised linear mixed models showing the relationship between the proportion of fatty acids in the blood and the proportion of sperm with a misshapen/abnormal head, in 59 greenfinches and 16 hawfinches (excluding three outliers). A single variable indicating the year, species and the country of collection was included as a random effect in all models. C:D = number of carbon atoms:double bonds, LCL = lower confidence limit, UCL = upper confidence limit. Significant results are presented in bold.

| Predictors	C:D	Response	df	z	p	r	LCL, UCL
Palmitic acid	C16:0	Misshapen sperm heads	71	-0.363	0.717	-0.04	-0.27, 0.19
Stearic acid	C18:0	Misshapen sperm heads	71	0.297	0.766	0.04	-0.19, 0.26
Oleic acid	C18:1 n-9	Misshapen sperm heads	71	1.460	0.144	0.17	-0.06, 0.38
Linoleic acid (LA)	C18:2 n-6	Misshapen sperm heads	71	-0.698	0.485	-0.08	-0.30, 0.15
Gamma-linolenic acid (GLA)	**C18:3 n-6**	**Misshapen sperm heads**	**71**	**-2.219**	**0.027**	**-0.25**	**-0.45, -0.03**
Dihomo-gamma-linolenic acid (DGLA)	C20:3 n-6	Misshapen sperm heads	71	1.035	0.301	0.12	-0.11, 0.34
Arachidonic acid (ARA)	C20:4 n-6	Misshapen sperm heads	71	-0.352	0.725	-0.04	-0.26, 0.19
Alpha-linolenic acid (ALA)	C18:3 n-3	Misshapen sperm heads	71	-0.281	0.779	-0.03	-0.26, 0.20
Eicosapentaenoic acid (EPA)	C20:5 n-3	Misshapen sperm heads	71	-0.784	0.433	-0.09	-0.31, 0.14
Docosapentaenoic acid (DPA)	C22:5 n-3	Misshapen sperm heads	71	-0.682	0.495	-0.08	-0.30, 0.15
Docosahexaenoic acid (DHA)	C22:6 n-3	Misshapen sperm heads	71	-0.662	0.508	-0.08	-0.30, 0.15
Total ω-6 PUFA		Misshapen sperm heads	71	-0.523	0.601	-0.06	-0.28, 0.17
Total ω-3 PUFA		Misshapen sperm heads	71	-0.552	0.581	-0.07	-0.29, 0.17
Ratio ω-6 / ω-3		Misshapen sperm heads	71	0.263	0.792	0.03	-0.20, 0.26
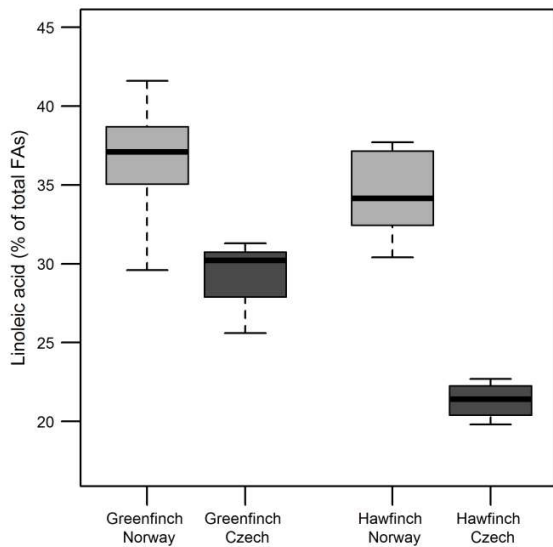
Figure 3. The level of linoleic acid (C18:2n-6, % of total fatty acids) in the blood of greenfinches from Norway (n = 63) and Czechia (n = 3), and hawfinches from Norway (n = 11) and Czechia (n = 8).
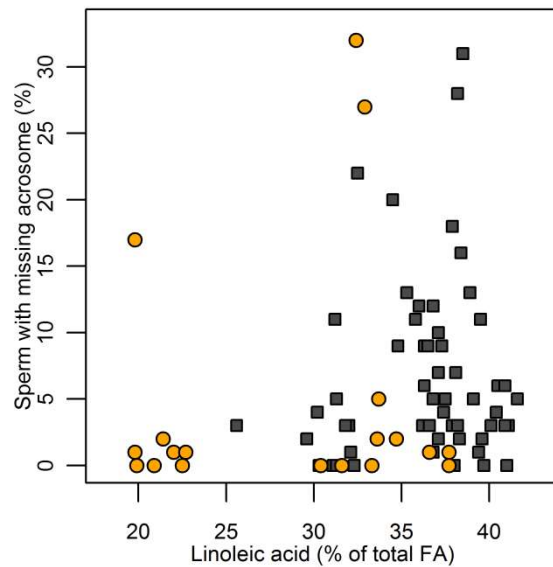

Figure 4. The relationship between the proportion of sperm with a missing acrosome (% of 100 sperm) and the proportion of linoleic acid (C18:2n-6, % of total fatty acids) in the blood of 58 greenfinches (dark grey squares) and 15 hawfinches (orange circles).

Discussion

143 We found that LA, a ω-6 PUFA, was the most abundant fatty acid in the blood of greenfinches and

144 hawfinches caught near sunflower feeders. In contrast, ω-3 PUFAs constituted a small proportion of

145 the total fatty acids, in particular for the greenfinch, where the ω-6/ω-3 ratio exceeded 75:1. There

146 was a positive correlation between the proportion of LA and the frequency of sperm with a missing

147 acrosome, suggesting a detrimental effect of LA-rich diets on reproductive health in wild finches,

148 although we found little evidence of an effect of LA on the frequency of misshapen sperm or on

149 sperm swimming speed.

150

151 LA constituted around a third of the total fatty acids in the blood of both greenfinches and

152 hawfinches, with particularly high levels for greenfinches sampled at bird feeders (36.6%). This is

153 relatively high compared to values reported in the literature for wild great tits (14-20% depending on

154 habitat and season, Andersson et al. 2015; Isaksson et al. 2015; Isaksson et al. 2017), as well as for

155 blue tits, tree sparrows, and house sparrows (10-20%, Isaksson et al. 2017), and for humans (23.5%,

156 Grimsgaard et al. 1999), although those data are from plasma rather than full blood. Similarly, the

157 levels of LA we found in the greenfinches and hawfinches were higher than for the six other sampled

158 species; in particular higher than the Parids (tits). Finches prefer to specifically visit sunflower feeders,

159 whereas Parids tend to consume many types of provided foods, such as fats. Moreover, birds from

160 feeder-dense areas in Norway had higher levels of LA than Czech birds, and the differences between

161 countries were larger than differences between species within a country. These results indicate that

162 fatty acid profiles are relatively labile, and are easily affected by food composition. This is interesting

163 in terms of comparative studies. Furthermore, LA is an essential fatty acid, meaning it must be

164 consumed through the diet and cannot be synthesised through metabolism. Together, these results

165 support the hypothesis that a sunflower-rich diet influences the fatty acid composition of the finches'

166 blood.

167   We found that the level of LA in the blood was positively correlated with the frequency of

168 sperm that were missing the acrosome. This relationship could potentially be related to oxidative

169 stress. As described in the introduction, LA can be metabolised into hydrogen peroxide, a reactive

170 oxygen species, which easily reacts with the unstable bonds of the unsaturated fatty acids in the

171 sperm membrane, causing sperm dysfunction (Aitken and Baker 2006; Brown 2016). Indeed, damage

172 of the acrosomal membrane can lead to premature acrosome reactions, and oxidative stress has

173 been shown to lead to premature acrosome reactions in rat sperm (Hsu et al. 1998). Thus, there is a

174 possibility that the sperm we observed had undergone premature acrosome reactions due to

175 damaged acrosomal membranes. Indeed, greenfinches have strongly helical sperm heads with wide,

176 exposed acrosomal membranes, and this type of cell morphology is associated with increased

177 frequency of abnormal sperm (Støstad et al. 2018a). Thus, it appears that the sperm of finches

178 feeding on LA-rich diets might be affected by oxidative stress. Tomášek et al. (2017) found that

179 oxidative challenge did not affect the number of abnormal sperm in zebra finches *Taeniopygia*

180 *guttata*, but they included sperm with tail deformities in their data, which may show different

181 responses than sperm heads to oxidative stress, and they also used diquat dibromide in the drinking

182 water rather than changing dietary fatty acid composition. Moreover, our results also show a

183 tendency for a negative correlation between EPA, a ω-3 PUFA, and the frequency of sperm with no

184 acrosome, which is consistent with previous literature; ω-3 PUFAs such as alpha-linolenic acid (ALA,

185 18:3n-3) have been shown to be associated with increased fertility in cockerels (Kelso et al. 1997)

186 and improved sperm motility in boars (Liu et al. 2017). Our results therefore indicate that excess

187 amounts ω-6 PUFAs, and possibly insufficient amounts of ω-3 PUFAs, could be detrimental for sperm

188 head integrity.

189   Intriguingly, we found a negative correlation between GLA, a ω-6 PUFA, and the proportion

190 of misshapen sperm heads. To our knowledge, there are no reports of any particular association

191 between avian (or human) sperm and GLA in the literature, and there is no measureable quantity of

192 GLA in sunflower seeds, so we cannot be certain of the cause of this correlation. However, the

193 proportion of GLA in our samples was relatively low, and sperm consist of both ω-3 and ω-6 PUFAs

194 (Cerolini et al. 2003); a deficiency of a necessary ω-6 PUFA can also potentially be detrimental for

195   sperm quality. As the fatty acid data are relative values rather than absolute values, there is also a

196   possibility that differences in the proportions of particular fatty acids in the blood was due to variable

197   amounts of other fatty acids, and/or that particular fatty acids had been deposited

198   disproportionately into other tissues. For example, low levels of GLA in the blood could mean high

199   levels of GLA in sperm or sperm-producing tissues (note that this is also the case for LA). For this

200   reason, future research could aim to also test the fatty acid composition of the sperm. There is also

201   the possibility that the correlation is statistically significant simply by chance, or related to an

202   unknown third factor.

203   The recommended dietary ω-6/ω-3 ratio for optimal fertility rate has been reported to about

204   6:1 to 9:1 for cockerels (Zanini et al. 2003). In contrast, the ω-6/ω-3 ratio of sunflower seeds is 329:1,

205   which far exceeds this recommendation. This means that a diet consisting solely of sunflower seeds

206   is unlikely to be optimal for reproductive function in wild birds, and indeed, the levels of sperm

207   abnormality we observed exceed levels that have been shown to negatively impact fertility in broiler

208   chickens (Wilson et al. 1979). Birds require several sperm for successful embryo development

209   (polyspermy, Hemmings and Birkhead 2015), and especially in a situation of intense sperm

210   competition, males are likely to experience a decrease in paternity success if the number of normal

211   sperm declines. Thus, there could be selection pressure for males to avoid over-consumption of food

212   items with excessive ω-6 PUFAs, such as sunflower seeds.

213   Interestingly, sunflower seeds are rich in vitamin E, which is considered to be an effective

214   antioxidant, and supplementation with antioxidants has been shown to lead to fewer sperm

215   abnormalities in zebra finches (Tomášek et al. 2017). However, our results indicate that the

216   antioxidants in sunflower seeds are not sufficient to counteract the negative influence of the

217   unbalanced ω-6/ω-3 ratio. Moreover, as excessive ω-6 PUFAs can increase inflammatory responses

218   (Cherian 2007), over-consumption may be detrimental to the general health status of individuals,

219   particularly for birds in challenging conditions, e.g. birds affected by disease. This is especially

220   relevant for greenfinches due to the current prevalence of the parasite *Trichomonas gallinae*

221   (Robinson et al. 2010) as well as the greenfinches' susceptibility to *Salmonella* infections (Refsum et

222   al. 2003; Grant et al. 2007). Considering that the average ω-6/ω-3 ratio in the blood of greenfinches

223   in our study was 76:1, it is possible that such high levels of ω-6 could aggravate the already poor

224   health condition of infected birds.

225   As we did not observe a statistically significant correlation between the proportion of any

226   fatty acid and sperm swimming speed, there appears to be little effect of fatty acid composition on

227   the functioning of the morphologically normal sperm. Alternatively, the abnormal sperm are still able

228 to swim normally, but given the correlation between sperm head shape and swimming speed

229 reported for songbirds (Støstad et al. 2018b), this is perhaps less likely. Unfortunately, we were not

230 able to acquire robust data for the proportion of motile sperm due to poor video/sample quality, but

231 previous studies show no association between fatty acid composition and sperm motility in poultry

232 (Khatibjoo et al. 2018) or in men (Conquer et al. 2000).

233   In summary, we demonstrate that urban finches have high blood levels of LA (a ω-6 PUFA),

234 possibly caused by consumption of LA-rich sunflower seeds. Our results also indicate that such high

235 levels of LA may be detrimental for sperm quality in these finches. We propose that the mechanism

236 could be membrane damage due to oxidative stress and subsequent premature acrosome reactions;

237 however, we encourage further research in this area to experimentally assess a causal relationship

238 between a sunflower-rich diet and sperm quality, as well as exploring the physiological mechanisms.

239 Nevertheless, our results provide early indications that a uniform diet of sunflower seeds may be

240 detrimental for the reproductive health of finches. If this proves to be the case, conservation

241 managers should consider whether the rising popularity of bird feeding might be contributing to the

242 decline of greenfinch populations.

243

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