REPRODUCTION

RESEARCH

Modification of sperm fatty acid composition during epididymal maturation in bats

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Abstract

Biochemical properties of polyunsaturated fatty acids (PUFAs) are fundamental to sperm movements. Amongst all adjustments operated during epididymal maturation, sperm membrane lipid composition is remodelled. Specifically, the proportion of PUFAs usually increases from the caput towards the cauda epididymidis. In mammals, PUFAs are predominantly acquired through the diet, which can consequently impact male fertility. We aimed at analysing to what extent n-6 and n-3 PUFAs are incorporated into sperm in the Seba's short-tailed bat (*Carollia perspicillata*), and at demonstrating the effect of the sperm fatty acid composition on sperm mobility. We therefore provided food varying in fatty acid composition to males of *C. perspicillata* and measured the fatty acid composition and mobility traits in spermatozoa collected from the caput and cauda epididymides. We found that n-6 and n-3 PUFAs and saturated fatty acids were significantly related to sperm velocity but not to the proportion of progressive sperm (i.e. motility). Concomitant to an increase in sperm velocity, the level of fatty acid saturation increased from the caput to the cauda epididymidis, while the proportion of PUFAs remained similar along the epididymis. A reduction in n-6 PUFAs counterbalanced an increase in n-3 PUFAs. The food treatments did not affect the sperm fatty acid composition. Our results suggest that a precise endogenous control rather than dietary effects determines sperm fatty acid composition in *C. perspicillata*.

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Introduction

In mammals, gametes have evolved anisogamously, with large oocytes and small motile sperm cells (Cummins & Woodall 1985). However, when they are released from the testes, spermatozoa are mostly immotile and require biochemical alterations provided during epididymal maturation in order to gain motility and the capacity to fuse with eggs (Aitken *et al*. 2007). Along the epididymis, these modifications are triggered by varying microenvironments generated by the epithelium in precise locations of the organ (Aitken *et al*. 2007, Gervasi & Visconti 2017).

In most species studied so far, mature sperm cells from the cauda epididymidis comprise a higher proportion of polyunsaturated fatty acids (PUFAs), and especially of n-3 PUFAs (also referred to as omega-3 fatty acids) in comparison to those from the caput (Parks & Hammerstedt 1985, Hall *et al*. 1991, Aveldaño *et al*. 1992, Haidl & Opper 1997, Pyttel *et al*. 2014, Angrimani *et al*. 2017). PUFAs possess molecular properties which increase membrane fluidity and influence transmembrane protein activities (Giroud *et al*. 2013, Pinot *et al*. 2014, Arnold *et al*. 2015).

The vigorous movements of mature sperm cells may require a flexible membrane and an elevated metabolic rate (Stubbs & Smith 1984, Lenzi *et al*. 1996, Kho *et al*. 2001, Ren *et al*. 2001, Storey 2008). Sperm velocity and motility have consistently been positively correlated to the PUFA content of sperm cells (Nissen & Kreysel 1983, Mourvaki *et al*. 2010). It has been repeatedly shown that n-3 PUFAs improve sperm quality significantly more than n-6 PUFAs (reviewed in Esmaeili *et al*. 2015). Nevertheless, it remains unclear why both types of PUFAs may differently impact sperm biology. PUFAs are essential to most mammal species in the sense that they are not produced *de novo* (Burr & Burr 1930) and should be acquired through the diet. After their incorporation, PUFAs can be elongated and desaturated to generate the long-chain PUFAs potentially missing in the diet. Human and livestock diets have been designed in order to improve sperm quality by modifying their lipid

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composition (Strzeżk *et al*. 2004, Brinsko *et al*. 2005, Estienne *et al*. 2008, Gliozzi *et al*. 2009, Mourvaki *et al*. 2010, Gulliver *et al*. 2012, Fair *et al*. 2014, Esmaeili *et al*. 2015, Maranesi *et al*. 2018).

Sperm competition, the post-copulatory competition of sperm from several males for the fertilisation of a given set of eggs (Parker 1970), can drive the evolution of faster sperm with higher rate of metabolism (Burness *et al*. 2004, Malo *et al*. 2005, Tourmente *et al*. 2013, Fitzpatrick & Lüpold 2014, Klemme *et al*. 2014). An efficient incorporation of PUFAs within the sperm membrane should thus have evolved in species facing post-copulatory competition to promote sperm competitiveness.

The mating system of Seba's short-tailed bat (*Carollia perspicillata*) is classified as a resource defence polygyny (Fleming 1988, McCracken & Wilkinson 2000). Harem males share reproduction with bachelor and peripheral males (Fasel *et al*. 2016) and males have relatively large testes (Orr & Zuk 2013). Both arguments suggest that sperm competition occurs in this species. *C. perspicillata* mostly feeds on the fruits of piper plant's (*Piperaceae*), but can also complete its diet with Arthropoda (Mello *et al*. 2004) or leaves (Pereira *et al*. 2018). Dietary fatty acid composition could thus vary. Changes in nutritional sources can potentially affect sperm membrane composition and post-copulatory competitiveness.

In this study, male *C. perspicillata* were given two dietary treatments differing in n-3 and n-6 PUFA content but similar in their overall PUFA proportion. The first aim was to measure the effects of the dietary treatments on the fatty acid composition of sperm cells collected from the caput and cauda epididymides. This will assess the incorporation of PUFAs within sperm cells along the epididymis. If dietary fatty acids were readily assimilated and unselectively incorporated in the sperm lipids, differences in the sperm fatty acid composition should reflect those of the food. A second aim was to measure the effects of the different fatty acid classes and of the location along the epididymis on *in vitro* sperm mobility traits. As PUFAs render membranes more flexible and increase cell metabolism, the proportion of PUFAs in sperm of *C. perspicillata* should be positively correlated with sperm mobility traits. Ultimately, epididymal maturation should lead to better sperm mobility in the cauda than in the caput epididymidis.

Materials and methods

Ethical statement

The veterinary office of the Canton Fribourg, after supervision of the Cantonal Ethical Committee, authorised the experimental setup and the detention conditions (2016_27_FR). Laboratory analyses were performed blind with respect to sample identity.

Animal housing

Bats were collected from a captive population, having free access to a tropical dome mimicking a Belizean dry forest (Papiliorama, Kerzers, Switzerland). Thirty *C. perspicillata* males were individually marked (PIT tags) and housed in five cages (dimensions: $1 \times 2 \times 2$ m), with six individuals per cage. Temperature ranged between 25 and 30°C and humidity reached more than 80%. The light-cycle was reversed and based on a 12L:12D cycle. Food consisted of mashed fruits and vegetables (apples, grapes, boiled carrots and bananas), complemented with vitamins, yeast, honey and grape sugar. Every night, 160 g of food was provided per cage during 6 h of the dark phase. This feeding regime simulated the conditions occurring during the dry season, when fruiting plants are sparse and rare. Most copulations in this species occur during the dry season (Fleming 1988). Water was provided *ad libitum*. All individuals were given a minimum of 1 week of acclimation to these conditions before the food treatments were started.

Experimental design

The experiment lasted 4 weeks. Males were equally and randomly assigned to one of the two treatments, hereafter named n-3 and n-6 PUFA treatments. One cage contained six males, three from each treatment. Twice a week, males of each cage were captured and separated in two different cages to feed on their respective food treatment. On the days of treatments, 1.1 mL of flaxseed oil (n-3 PUFA treatment) or 0.9 mL of sunflower oil mixed with 0.2 mL flaxseed oil (n-6 PUFA treatment) were added and thoroughly mixed to 80 g of the daily food ration. On some occasions ($N = 8$), we collected faeces after treatment and analysed the fatty acid composition. Compared to the n-3 PUFA treatment, faeces collected from bats feeding on the n-6 PUFA treatment contained a significantly larger proportion of n-6 PUFAs (linear mixed model with logit transformation of the response variable: estimate: 1.19 ± 0.26, $F_{1,6} = 21.29$, $P = 0.004$, $R^2 = 0.78$) and a significantly lower proportion of n-3 PUFAs (linear mixed model with logit transformation of the response variable: estimate: -1.50 ± 0.41, $F_{1,6} = 13.27$, $P = 0.011$, $R^2 = 0.69$).

Sperm collection

After 4 weeks of treatment, males were killed via an intraperitoneal overdose of sodium pentobarbital and an orchiectomy was performed. Epididymides were separated from the testes and blood vessels were removed. Caput and cauda epididymidis were separately minced in 100 µL of HEPES buffer solution (HBS: 150 mM NaCl, 5 mM HEPES, pH 7.4). Sperm were filtered using a 30 µm filter (Partec, Münster, Germany) and rinsed with 100 µL of HBS. From the mix, 3 µL was used for the mobility analysis. The mix was then centrifuged (600 ***g***, 5 min) to remove the supernatant and washed twice with 200 µL of HBS. In samples with a visible pellet, the pellet was finally suspended in 50 µL of HBS and split in two tubes. Only 25 µL of HBS were added to samples with a lower amount of sperm and those were not split. Finally, 25 µL of butylated hydroxytoluene (BHT, antioxidant, 2 mg/mL) diluted in 100%

MeOH were added to the washed sperm samples. Samples were then placed on dry ice until reaching the lab where they were frozen at −70°C until analysis.

Sperm mobility analysis

Sperm motion was video-recorded using a Kappa CF 8/5 camera mounted on an Olympus XK41 microscope set with 200× magnification under dark-field conditions. Three microlitres of filtered sperm (i.e. before the centrifugation) were injected in a 20-µm-deep chamber slide (SC 20-01-04-B, Leja, Netherlands) within 10 min after dissection. Three 2-s videos at a frame rate of 50 Hz were then analysed for each sample using a CASA plug-in in ImageJ 1.47v (Rasband, National Institute for Health, USA; Wilson-Leedy & Ingermann 2007) to measure sperm velocity (i.e. curvilinear velocity (VCL), µm/s) of progressive sperm cells. As previously reported (Fasel *et al.* 2015), measures of VCL significantly correlated to the other swimming parameters (Table 1) calculated by CASA, with the single exception of the linearity (NJ Fasel 2018, unpublished data). Sperm motility (i.e. the proportion of progressive sperm) was estimated subjectively by a unique observer (KMc) and significantly correlates to the CASA estimation (linear model with CASA estimation as explanation of subjective measures, the intercept forced to zero and both variables arcsine square root transformed: estimate: 0.87 ± 0.04, $F_{1,131} = 451.43$, $P < 0.001$, $R^2 = 0.79$). Finally, the mean of the mobility measures per sample (three videos) was calculated.

Fatty acid analysis

Fatty acid methyl esters (FAMEs) were prepared by incubating samples (20 µL) with 1 mL of 5% (v/v) H_2SO_4 in MeOH in the presence of 0.1% (w/v) BHT. The transesterification reaction was performed in a dry block heater (VWR, Dietikon, Switzerland) at 85°C for 30 min. At the end of the reaction, tubes were cooled down to room temperature, briefly spun down, and 1.5 mL of 0.9% (w/v) NaCl and 2 mL of *n*-hexane were added to the solution. The mixture was strongly shaken for 5 min and both organic and hydroalcoholic phases were separated by centrifugation at 1500 ***g*** for 5 min. The upper organic (*n*-hexane) phase was transferred into a new glass tube. The *n*-hexane phases were evaporated under a nitrogen flux and FAMEs were re-suspended in 30 µL of *n*-hexane. FAME samples were transferred into 0.2 mL crimp vials (BGB Analytik, Genève, Switzerland) prior to injection into the gas separator to perform gas chromatography (GC).

FAMEs were analysed using a gas chromatograph coupled to a flame ionisation detector (Agilent 7890A). FAME samples dissolved in *n*-hexane were introduced to the injection port heated to 250°C with an automated liquid sampler (Agilent 7993). The samples were injected without splitting. FAMEs were separated on a 30-m long × 0.25-mm ID × 0.25 µm DB-23 capillary column (Agilent) using He as vector gas (2.6 mL/min). The oven temperature, initially set to 100°C for 2 min, was first increased to 160°C at 25°C/min, then to 250°C at 8°C/min and maintained at this temperature for an additional 4 min. The detector temperature was set to 270°C while detector gases were set to 30 mL/min for H_2, 400 mL/min for air, and 30 mL/min for makeup gas (He). Data were recorded at a frequency of 50 Hz. FAME quantifications were performed with calibration curves built with the Supelco 37 component FAME mix (Sigma-Aldrich) using 17:0 methyl ester as the internal standard (250 ng). FAME determination was based on the retention times of each component and compared with those of FAMEs contained in Supelco 37 FAME mix (Sigma-Aldrich) and of 22:5 n-3 and 22:5 n-6.

Statistical analysis

The different types of FAMEs were classified as PUFAs, monounsaturated (MUFA) or saturated fatty acids (SFAs). Within the PUFA class, the n-6 and n-3 PUFAs were distinguished. The proportions of each fatty acid class over the total amount of FAMEs measured were calculated. Analyses were conducted with R (version 3.3.2).

First the effects of the treatments and of the location in the epididymis (caput vs cauda) on the sperm fatty acid composition were estimated. The response variables (i.e. fatty acid class proportions) were logit transformed. Then, to account for the small sample size and the large variance in some fatty acid classes, robust linear mixed models were run (function: rlmer, package: robustlmm; Koller 2016). The null hypothesis was rejected when the 95% CI of an effect estimation did not include 0. As explanatory variables, the treatments, the parts of the epididymis and the interaction between them were considered. The cage number and the identity of the male, nested within the cage number, were considered as random factors.

Secondly, the effects of the sperm fatty acid classes and of the location in the epididymis on sperm velocity and motility were evaluated. VCL and the subjectively estimated motility were used as response variables. Motility was transformed with arcsine square root, which is more appropriate than the logit

Table 1 Mobility traits in *C. perspicillata* sperm from caput (CAP) and cauda (CAU): CASA motility (MOT, proportion), subjective motility (MOTs, proportion), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), progression (PROG, µm), beat cross frequency (BCF), wobble (WOB) and linearity (LIN, VSL/VAP).

	MOT	MOTs	VCL	VSL	VAP	PROG	BCF	WOB	LIN
CAP									
Mean	0.056	0.056	26.667	7.270	11.202	15.397	23.388	0.376	0.661
S.E.	0.084	0.082	13.264	5.856	7.810	10.596	8.122	0.115	0.146
CAU									
Mean	0.089	0.083	32.832	12.388	15.494	16.863	22.839	0.413	0.754
S.E.	0.080	0.111	13.753	8.389	9.715	8.396	6.486	0.121	0.108

Means and standard errors (S.E.) are reported.

transformation when data contain 0-values. As explanatory variables, the proportions of the classes of fatty acids (n-6 and n-3 PUFAs, MUFAs and SFAs) were analysed individually in four different models. Combining all classes of fatty acids in one single model would not have been possible because of collinearity. The part of the epididymis and its interaction with the fatty acid classes were also included as explanatory variables. Logit-transformed proportions were centred around the mean values per epididymis in order to interpret the effect of the epididymis part as a main effect but also in interaction with the classes of fatty acids. Male identity and cage number, with male identity nested within cage number, were considered as random factors. Robust linear mixed models (function: rlmer, package: robustlmm) were run. The null hypotheses were rejected when the 95% CI of an effect estimation did not include 0. For all above analyses, non-significant interactions were discarded (Engqvist 2005).

Results

The interaction between the treatment and the epididymis parts had never a significant effect on the proportions of n-3 (CI: −0.359 to 0.228) and n-6 PUFA (CI: −0.229 to 0.409), MUFA (CI: −0.398 to 0.163) and SFA (CI: −0.036 to 0.281) in the sperm cells and was consequently discarded from the models. The food treatments did not trigger significant changes in any of the fatty acid classes (Table 2). Sperm from the cauda epididymidis had higher proportion of n-3 PUFAs and SFAs, and a lower proportion of n-6 PUFAs and MUFAs in comparison to those from the caput part (Fig. 1). However, the proportion of total PUFAs did not vary along the epididymis (Table 2). The mean percentage and standard deviation for each fatty acid type and class are listed in Table 3.

Sperm VCL was not affected by the different classes of fatty acids in interaction with the part of the epididymis: n-3 (CI: −33.482 to 2.314) and n-6 PUFAs (CI: −31.870 to 13.115), MUFAs (CI: −27.806 to 39.775) and SFA (CI: −21.867 to 30.229). Those interactions were consequently removed from the final models. The proportions of n-3 and n-6 PUFAs were positively correlated with sperm VCL (Fig. 2), while the proportion of MUFAs did not significantly affect sperm VCL. Finally, the proportion of SFAs affected negatively this variable (Fig. 2 and Table 4). Sperm VCL was consistently significantly higher in the cauda epididymidis (Table 4). Analysed individually, the different classes of fatty acids did not affect sperm motility in interaction with the part of the epididymis: n-3 (CI: −0.417 to 0.214) and n-6 PUFAs (CI: −0.638 to 0.039), MUFAs (CI: −0.233 to 0.722) and SFA (CI: −0.295 to 0.533). Those interactions were consequently removed from the final models. The different proportions of fatty acid classes were not correlated with sperm motility (Table 4). Finally, the sperm collected from the cauda epididymidis were not more motile than those from the caput (Table 4).

Table 2 Effects of the dietary treatment and epididymis part on the proportion of the various fatty acid classes in sperm.

	Estimate	SE	CI
n-3 PUFA (logit)			
Intercept	−1.990	0.092	−2.169 to −1.810
Treatment n-6 PUFA	−0.110	0.075	−0.257 to 0.038
Cauda epididymidis	0.359	0.074	0.214 to 0.504
n-6 PUFA (logit)			
Intercept	−1.387	0.082	−1.547 to −1.227
Treatment n-6 PUFA	0.154	0.080	−0.004 to 0.311
Cauda epididymidis	−0.211	0.080	−0.368 to −0.056
PUFA (logit)			
Intercept	−0.747	0.101	−0.945 to −0.548
Treatment n-6 PUFA	0.059	0.080	−0.097 to 0.215
Cauda epididymidis	0.049	0.079	−0.105 to 0.203
MUFA (logit)			
Intercept	−1.274	0.069	−1.410 to −1.138
Treatment n-6 PUFA	0.030	0.079	−0.125 to 0.184
Cauda epididymidis	−0.364	0.079	−0.519 to −0.209
SFA (logit)			
Intercept	−0.128	0.041	−0.209 to −0.048
Treatment n-6 PUFA	−0.034	0.047	−0.126 to 0.057
Cauda epididymidis	0.101	0.047	0.010 to 0.193

Estimates, standard errors (S.E.) and 95% confidence intervals (CI) obtained with robust linear mixed models are given. The interaction terms were always not significant and were therefore removed from the final model.

Discussion

In this study, we investigated to what extent sperm fatty acid composition can be affected by varying dietary n-6 and n-3 PUFA proportions in *C. perspicillata*. Furthermore, we described the changes in the fatty acid composition of the sperm lipids along epididymal maturation. Finally, we estimated the effects of fatty acid composition on sperm velocity and motility and its development along the epididymis.

Our results revealed that the transit through epididymis resulted in a higher swimming speed of the motile sperm recovered from cauda compared to caput epididymidis (Fig. 1), indicating sperm maturation. A higher velocity may reduce the time in the vagina and the uterus, where conditions are deleterious to sperm (Rasweiler *et al*. 2010). Despite the increase in velocity,

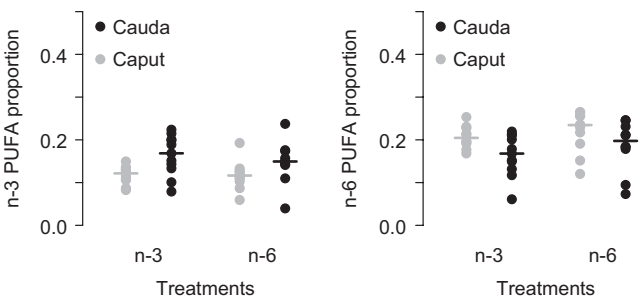

Figure 1 Proportion of PUFA in the sperm membrane from *C. perspicillata*. Median values per dietary groups (n-3 vs n-6 PUFAs) and per epididymis parts (caput and cauda) are indicated with a segment.

Table 3 Percentage of the different fatty acids types and classes over the total amount of fatty acid methyl esters (FAMEs) measured in two parts of the epididymis of *C. perspicillata*, following 4 weeks of n-3 or n-6 PUFAs supplementation.

	n-3 PUFA treatment, Mean ± S.D.		n-6 PUFA treatment, Mean ± S.D.	
	Caput	Cauda	Caput	Cauda
16:0	37.81 ± 0.89	37.16 ± 4.47	36.02 ± 5.89	36.96 ± 3.68
16:1	0.60 ± 0.70	0.46 ± 0.72	0.86 ± 0.60	0.15 ± 0.33
18:0	6.70 ± 1.46	11.13 ± 3.01	5.89 ± 2.22	10.50 ± 2.39
18:1 n-9	16.46 ± 3.41	13.34 ± 6.39	14.71 ± 5.70	11.88 ± 4.70
18:1 n-11	4.51 ± 0.49	4.18 ± 1.54	5.80 ± 4.88	5.18 ± 0.58
18:2 n-6	0.47 ± 0.63	1.38 ± 0.73	0.78 ± 0.59	1.29 ± 0.92
18:3 n-6	n.d.	n.d.	0.43 ± 1.41	n.d.
18:3 n-3	n.d.	n.d.	0.87 ± 2.89	n.d.
20:0	1.45 ± 0.55	0.76 ± 0.80	1.46 ± 1.25	1.95 ± 2.51
20:1	0.03 ± 0.11	n.d.	1.38 ± 4.37	n.d.
20:2 n-6	0.16 ± 0.39	n.d.	0.47 ± 0.62	n.d.
20:3 n-6	0.43 ± 0.56	0.73 ± 0.71	0.63 ± 0.52	0.66 ± 0.70
20:4 n-6	15.39 ± 2.06	9.04 ± 1.92	16.36 ± 2.59	11.60 ± 3.35
20:3 n-3	n.d.	n.d.	0.21 ± 0.71	n.d.
20:5 n-3	0.04 ± 0.14	n.d.	0.25 ± 0.55	n.d.
22:0	n.d.	n.d.	0.05 ± 0.18	n.d.
22:1	n.d.	0.19 ± 0.64	0.06 ± 0.20	n.d.
22:4 n-6	1.51 ± 0.95	2.12 ± 2.12	1.32 ± 1.04	2.17 ± 2.22
22:5 n-6	2.70 ± 2.38	3.11 ± 3.38	2.03 ± 1.45	2.87 ± 2.82
22:6 n-3	1.39 ± 0.99	1.09 ± 0.75	1.40 ± 0.83	1.15 ± 1.19
24:0	10.30 ± 2.29	15.30 ± 4.60	8.95 ± 2.19	13.63 ± 4.89
24:1	0.04 ± 0.12	n.d.	0.06 ± 0.20	n.d.
PUFA	32.40 ± 3.56	32.78 ± 8.59	33.71 ± 7.22	33.37 ± 9.74
MUFA	21.64 ± 3.44	18.18 ± 6.29	22.86 ± 2.84	17.22 ± 4.85
SFA	45.96 ± 1.85	49.05 ± 7.02	43.43 ± 7.91	49.41 ± 6.80
n6	20.66 ± 2.61	16.38 ± 4.84	22.02 ± 4.76	18.59 ± 5.92
n3	11.73 ± 2.18	16.39 ± 4.70	11.69 ± 3.27	14.77 ± 5.03

Means and standard deviations (S.D.) are reported.

the proportion of motile sperm in samples collected from the cauda epididymidis was surprisingly not higher than in samples from the caput. This result contrasts with those from other species (Dacheux & Dacheux 2014), bats included (Rodríguez-Tobón *et al*. 2015). Mobility measures were recorded with sperm not swimming in seminal fluids but in HEPES buffer. The latter was not designed for sperm mobility analyses but allowed trait comparisons without influencing further lipid analyses. This sub-optimal buffer solution may explain the measures of motility and velocity lower than expected (Fasel *et al*. 2015).

The modification of the sperm membrane is one factor believed to allow sperm to gain in velocity. Accordingly, we showed that the proportions of both n-3 and n-6 PUFAs were positively correlated to sperm velocity in *C. perspicillata* (Fig. 2), corroborating results found in other species (Mourvaki *et al*. 2010, Maranesi *et al*. 2018). PUFAs may increase the activity of trans-membrane proteins (Giroud *et al*. 2013, Arnold *et al*. 2015), which would then favour sperm movements (Stubbs & Smith 1984, Kho *et al*. 2001, Ren *et al*. 2001, Storey 2008). In addition, PUFAs' lower melting temperatures may provide sperm with a more flexible membrane, allowing more effective propulsion (Mortimer 1997, Pinot *et al*. 2014). The negative correlation between SFA and VCL further supports that sperm with a membrane containing less saturated fatty acids are faster (Fig. 2). However, the concomitant increases in VCL and in SFA proportion measured along the epididymis recalls that other factors than the membrane fatty acid composition are also involved in sperm maturation and control sperm velocity (e.g. enhanced metabolism, morphological and osmolarity changes, protein incorporation, environmental molecular composition, etc.).

Changes in sperm fatty acids composition was shown to occur during epididymal maturation through the incorporation of lipids from the organ microenvironment (Parks & Hammerstedt 1985, Hall *et al*. 1991, Aveldaño *et al*. 1992, Haidl & Opper 1997, Pyttel *et al*. 2014, Angrimani *et al*. 2017). Our measures in *C. perspicillata* confirmed that a change in the PUFA composition occurred during the passage from the caput to the cauda epididymidis, with a partial replacement of the n-6 PUFAs by the n-3 PUFAs, without any significant change in the overall PUFA proportion. The importance of n-3 PUFAs in the performance of mammalian sperm has been repeatedly supported (reviewed in Esmaeili *et al*. 2015). An increase in the proportion of n-3 PUFAs during the last stages of epididymal maturation is consequently not surprising.

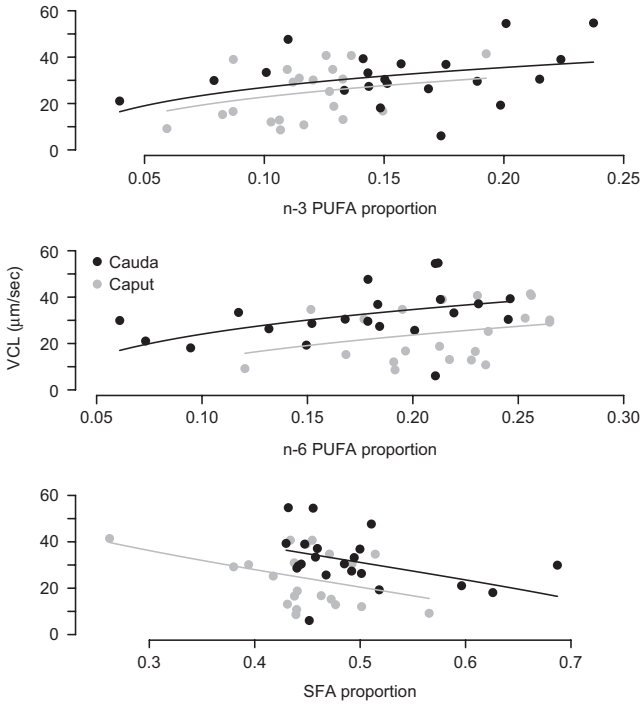

Figure 2 Curvilinear velocity (VCL) of caput and cauda sperm of *C. perspicillata* related to the n-3 PUFA, n-6 PUFA and SFA proportions over the total amount of FAMEs in the sperm cells.

The observed maintenance in overall PUFA proportion can indicate a need to keep sperm membrane resistant to oxidative stress. Indeed, besides adjustments of antioxidant defences in response to external stressors (Losdat *et al*. 2014, Rojas Mora *et al*. 2017), the fatty acid composition of the sperm membrane could be adjusted to its environment: species whose ejaculate will face elevated ROS levels might reduce the PUFA content of sperm membrane (delBarco-Trillo & Roldan 2014, delBarco-Trillo *et al*. 2015). Ejaculates from *C. perspicillata* display low lipid peroxidation markers (Fasel *et al*. 2017). This further supports that the proportion of PUFAs within sperm cells is under control during epididymal maturation to deal with an elevated metabolism and high ROS levels. Additionally, the measured reduction in MUFAs and the increase in SFA in the cauda epididymidis could further strengthen oxidative stress resistance. In sperm cells collected from the caput and cauda epididymidis of *C. perspicillata*, low but increasing amounts of plasmalogens were detected (U Jakop, NJ Fasel, L Méné-Saffrané, J Schiller, K Müller & KM Engel 2018, unpublished data). In these phospholipids, a SFA is generally fixed to the sn-1 position through an ether bond (Lenzi *et al*. 1996, Pyttel *et al*. 2014). Consequently, from sn-1 position no FAME was generated. This would even lead to a slight underestimation of SFAs along the epididymides.

Table 4 Effects of the proportion of the various fatty acid classes in sperm and of the epididymis part on sperm velocity and motility in *C. perspicillata*.

	Estimate	SE	CI
Velocity			
Intercept	24.551	4.292	16.139 to 32.962
n-3 PUFA	14.762	4.674	5.602 to 23.923
Cauda epididymidis	7.294	3.113	1.194 to 13.395
Intercept	24.464	3.209	18.174 to 30.754
n-6 PUFA	11.789	5.014	1.962 to 21.616
Cauda epididymidis	7.361	3.443	0.612 to 14.109
Intercept	24.395	3.573	17.393 to 31.398
MUFA	−7.397	7.098	−21.309 to 6.515
Cauda epididymidis	7.214	3.681	0.001 to 14.428
Intercept	24.306	3.391	17.659 to 30.953
SFA	−18.251	6.807	−31.593 to −4.909
Cauda epididymidis	7.379	3.313	0.885 to 13.872
Motility			
Intercept	0.175	0.039	0.098 to 0.251
n-3 PUFA	0.122	0.074	−0.023 to 0.267
Cauda epididymidis	0.047	0.047	−0.045 to 0.138
Intercept	0.168	0.038	0.094 to 0.243
n-6 PUFA	0.039	0.076	−0.110 to 0.188
Cauda epididymidis	0.052	0.054	−0.053 to 0.158
Intercept	0.168	0.038	0.094 to 0.243
MUFA	−0.080	0.099	−0.274 to 0.114
Cauda epididymidis	0.053	0.054	−0.053 to 0.158
Intercept	0.168	0.038	0.094 to 0.243
SFA	−0.057	0.104	−0.262 to 0.148
Cauda epididymidis	0.052	0.054	−0.053 to 0.158

Motility values were arcsin-square root transformed. Estimates, standard errors (S.E.) and 95% confidence intervals (CI) obtained with robust linear mixed models are given. The interaction terms were always not significant and were therefore removed from the final models.

In their comparative analysis, delBarco-Trillo and Roldan (2014) did not include any bat species and the proportion of PUFAs in sperm measured in *C. perspicillata* would be the second lowest of the 21 species considered in that analysis. The sperm of some flying foxes have been shown to contain extremely low proportions of PUFAs (Melville *et al*. 2012). The low proportions of PUFAs in the sperm of the few bats investigated so far could be related to an intense sperm competition (Orr & Zuk 2013), favouring increased sperm velocity but at the same time leading to a rise in ROS generation due to the boosted energy production (Burness *et al*. 2004, Tourmente *et al*. 2013, Fitzpatrick & Lüpold 2014, Klemme *et al*. 2014). Alternatively, high SFA proportions in bat sperm could be related to the capacity of many bat species to store sperm (Orr & Zuk 2013). Indeed, a similar pattern was already shown in the sperm of bees which can be stored up to several years (Wegener *et al*. 2013). It remains nevertheless unclear whether the capacity to store sperm has been lost in species like *C. perspicillata* or if its evolution is polyphyletic.

Ultimately, the variation in the dietary PUFAs did not affect the composition of the sperm neither in the caput

nor in the cauda epididymidis (Fig. 2). The food delivered during the treatment days were expected to comprise approximately 70% of the weekly fat intake. The gut transit time in *C. perspicillata* is short (Bonaccorso & Gush 1987, Laska 1990), and the efficiency to extract lipids might be low. If the bats only incorporated a limited amount of fat during the treatment days, the effect of the dietary treatment on the sperm fatty acid composition might have been reduced. However, as already mentioned above, the proportion of PUFAs in mature sperm (i.e. in the cauda epididymidis) was low in comparison to other mammalian species investigated so far (delBarco-Trillo & Roldan 2014). Therefore, the absence of any significant effects of our dietary treatments may alternatively be explained by the need to maintain the sperm resistant to oxidative stress. Furthermore, feeding habits of *C. perspicillata* have been shown to vary seasonally (Mello *et al*. 2004, Pereira *et al*. 2018). As sperm production is maintained throughout the year (Fleming 1988), epididymal lipid metabolism has probably evolved to resist to the likely seasonal variation in dietary fatty acid composition.

In conclusion, our study describes for the first time the changes in the fatty acid composition of the sperm during epididymal maturation in a bat species. Furthermore, we highlight the relation between the fatty acid composition of the sperm and sperm velocity. This link is often mentioned but has rarely been established.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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