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1 **Short Communication: Long-term intake of the illegal diet pill DNP reduces**
2 **lifespan in a captive bird model**

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22 **Keywords**

23 2,4-dinitrophenol, toxicity, mitochondrial uncoupling, oxidative stress, survival, longevity.

24

25

26 **Abstract**

27 2,4-Dinitrophenol (DNP), a molecule uncoupling mitochondrial oxidative phosphorylation from oxygen
28 consumption, is illegally used by humans as a diet pill, but is nonetheless investigated as a potential
29 human medicine against 'metabesity'. Due to its proven acute toxicity and the scarceness of long-term
30 studies on DNP administration in vertebrates, we determined the impact of a long-term DNP treatment
31 ($\sim 4 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, *i.e.* within the range taken illegally by humans) on body mass, metabolism, ageing
32 and lifespan in a captive bird model, the zebra finch. The chronic absorption of DNP over life (>4 years)
33 led to a mild increase in energy expenditure (*ca.* +11% compared to control group), without
34 significantly altering the normal slight increase in body mass with age. DNP did not significantly
35 influence the alteration of physical performance, the rise in oxidative damage, or the progressive
36 shortening of telomeres with age. However, DNP-treated individuals had a significantly shorter
37 lifespan (*ca.* -21% in median lifespan compared to control group), thereby raising potential concerns
38 about DNP use as a diet pill or medicine.

39 **Introduction**

40 There is much public and academic interest in discovering human nutritional supplements that
41 increase fat metabolism and so promote body mass loss (Jeukendrup and Randell, 2011). One example
42 of those substances is 2,4-Dinitrophenol (DNP), an industrial product that was found to trigger body
43 mass loss when accidentally inhaled by factory workers in the 1930s (Harris and Cocoran, 1995). Early
44 scientific studies established that DNP is an efficient means to promote body mass loss, but its acute
45 toxicity was quickly revealed, culminating in many fatalities and the prohibition of its usage as human
46 medicine (Harris and Cocoran, 1995). However, DNP made its comeback in recent years, being
47 marketed and sold illegally through the internet and social media (Ainsworth et al., 2018; McVeigh et
48 al., 2017). This led to a marked increase in DNP usage and its associated risks, culminating in several
49 fatalities per year in the last decade (Grundlingh et al., 2011; Hoxha and Petroczi, 2015).

50 DNP promotes body mass loss through a partial uncoupling of the oxidative phosphorylation
51 (ATP production) system in mitochondria (Harris and Cocoran, 1995). When uncoupled, mitochondria
52 are less efficient in converting energy and use more fuel to provide an equivalent amount of ATP
53 (Brand, 2000). Concomitantly, mild mitochondrial uncoupling has the potential to reduce reactive
54 oxygen species (ROS) production by the mitochondria, and thus to prevent oxidative stress and to
55 extend lifespan according to the *uncoupling to survive hypothesis* (Brand, 2000). Experiments using
56 DNP in various eukaryotic models (see Table 1) mostly support the *uncoupling to survive hypothesis*.
57 However, DNP induces mitochondrial heat production, thereby making results from ectotherms (Table
58 1) potentially difficult to translate to endotherms, including humans. Additionally, the beneficial
59 effects observed in mice (*i.e.* increased longevity, improved glucose-insulin-triglycerides plasma levels,
60 decreased oxidative stress levels; Caldeira da Silva et al., 2008) might be associated with the anti-
61 obesity effect of DNP in this species. Such beneficial effects might thus be absent in animal models not
62 displaying age-related obesity or in non-obese humans. Despite its known toxicity (Harris and Cocoran,
63 1995), DNP has recently been granted an open Investigation New Drug (IND) approval by the FDA to
64 begin clinical testing linked to its potential to prevent 'metabesity' (*i.e.* global comorbidities associated

65 with the over-nutritional phenotype; Geisler, 2019). Therefore, it seems timely to evaluate the
66 potential effects of chronic DNP treatment on ageing and lifespan using endotherm models not
67 displaying age-related obesity.

68 We previously highlighted that medium-term (*i.e.* 1 month) DNP chronic treatment at a dose
69 of $\sim 4 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (*i.e.* within the range taken illegally by humans; Table 1) had the expected
70 stimulating effect on metabolic rate in captive zebra finches (*Taeniopigya guttata*), but was mainly
71 compensated by a corresponding increase in food intake (Stier et al., 2014). In the present article, we
72 use long-term data collected on the same birds to test the effects of DNP on lifelong body mass
73 dynamics, ageing markers and lifespan of individuals followed over > 4 years of treatment.

74

75 **Material & Methods**

76 As explained in details in Stier et al. (2014), 60 captive zebra finches (32 females and 28 males)
77 were randomly allocated to either a control group, or an experimental group treated with $\sim 4 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$
78 of DNP from 0.75 to 5.2 years of age. DNP treatment was administrated through the drinking
79 water and did not result in any alteration of water intake (Stier et al., 2014). The DNP dose was chosen
80 as the lowest dose eliciting an increase of whole-body metabolic rate (Stier et al., 2014). Individuals
81 were followed longitudinally over the course of their life. Specifically, we measured body mass and
82 collected blood samples at 11, 14, 24, 34 and 58 months of age. We estimated average metabolic rate
83 at 12 and 24 months of age as overnight VO_2 (see Stier et al. (2014) for details), while also recording
84 fasting body mass loss during the metabolic measurement (~ 10 hours) normalized to 24 hours (*i.e.*
85 expressed in $\text{g}\cdot\text{day}^{-1}$). We assessed vertical flight speed at 12.5 and 25 months of age following Reichert
86 et al. (2015) as an indicator of physical performance and used its decline with age as a biomarker of
87 ageing. We measured two biomarkers of ageing from blood samples. First, we measured oxidative
88 damage as plasma reactive oxygen metabolites (ROMs) (see Stier et al. (2014) for details). Indeed,
89 oxidative damage levels in the blood have been shown to increase with age, including in captive zebra
90 finches (Marasco et al., 2017), and high levels of plasma ROMs have been associated with increased

91 mortality risk in humans (Schöttker et al., 2015). Second, we measured relative telomere length of
92 blood cells using qPCR (see Reichert et al. (2014) for details). Indeed, telomeres usually shorten with
93 age, and short telomeres have been shown to predict increased mortality risk, including in captive
94 zebra finches (Heidinger et al., 2012).

95 Control and DNP-treated birds did not statistically differ before the start of the treatment in
96 terms of body mass, metabolic rate or oxidative damage (see Stier et al. (2014) for details). Statistical
97 analyses were conducted using SPSS 20.0. Metabolic rate, body mass, fasting body mass loss, ROMs
98 and telomere length were analyzed using general estimating equations (GEEs) with bird identity as a
99 random factor, and DNP treatment, Age and Sex as fixed factors (see details in Table 2). Additional
100 covariates were added to specific models, such as body mass for the metabolic rate model and pre-
101 treatment telomere length for the telomere model (see details in Table 2). Survival was analyzed using
102 a Cox regression with DNP treatment as fixed factor, with 20 individuals still alive at the end of the
103 study (*i.e.* 14 control vs. 6 DNP) being censored.

104

105 **Results and Discussion**

106 Chronic DNP treatment induced a moderate increase in energy expenditure that was
107 consistent over time (*ca.* +11% compared to control group, Fig. 1A, Table 2A), confirming that our DNP
108 dose induced a temporally stable mild uncoupling. However, DNP did not significantly influence the
109 expected slight increase in body mass observed with age (Fig. 1B, Table 2B), but it increased body mass
110 loss during fasting (Fig. 1C, Table 2C), which is in line with its effect on metabolic rate. DNP did not
111 appear to protect birds from the degradation of their locomotor performances with increasing age,
112 since the average flight speed decreased similarly in both control and DNP-treated birds between 12.5
113 and 25 months of age (Fig. 1D, Table 2D).

114 Ageing is a multifactorial process among which mitochondrial dysfunction, the accumulation
115 of oxidative damage and the shortening of telomeres are suggested to play a role (López-Otín et al.,
116 2013). DNP did not significantly prevent the age-related increase in oxidative damage levels over a

117 period of *ca.* 4 years (Fig. 1E, Table 2E), confirming our previous results in early adulthood (Stier et al.,
118 2014). Telomere length and shortening rate are thought to play a causal role in the ageing process
119 (Muñoz-Lorente et al., 2019). Yet, we found no significant effect of chronic DNP exposure on telomere
120 length or the age-related telomere shortening (Fig. 1F, Table 2F), suggesting no protective or
121 detrimental effects of mild mitochondrial uncoupling on cellular ageing rate. Finally, our study
122 highlights an overall detrimental effect of chronic DNP treatment on lifespan (median lifespan: DNP =
123 1420 days, Control = 1803 days; $B = -0.66 \pm 0.32$, Wald $\chi^2 = 4.17$, $p = 0.041$, Fig. 1G), a result in complete
124 contradiction with previous experiments in other eukaryotic models (Table 1).

125 This disparity with previously published results could hypothetically be linked to specificities
126 in avian physiology and life-history. For instance, birds differ from mammals in terms of longevity,
127 being typically long-lived for their body size (Holmes et al., 2001). Zebra finches have a typical median
128 lifespan of approximately 3-5 years (*e.g.* ~ 3 years in Marasco et al. 2017; ~ 4 years in Briga et al. 2019;
129 ~ 5 years in the present study for control birds), being therefore longer-lived than laboratory mice (~ 2
130 years in Caldeira da Silva et al., 2008). Humans and birds being long-lived for their body size, some
131 authors suggested that birds could be better models to understand human ageing than traditional
132 short-lived rodents (Holmes and Ottinger, 2003). On another note, we have previously shown that the
133 sensitivity of *in vitro* mitochondrial ROS was lower in zebra finch than in laboratory mouse (Stier et al.,
134 2014), which could contribute to explain the difference between results on mice (Caldeira da Silva et
135 al., 2008) and zebra finches (this study). Yet, our results suggest that deleterious effects of chronic DNP
136 intake could occur and calls for further studies using long-term DNP treatment in other endotherm
137 models that do not necessarily display age-related obesity.

138 Our study highlights that, even at a moderate dose (*i.e.* increasing metabolic rate by only *ca.*
139 11%), a chronic DNP treatment can shorten lifespan. DNP promotes proton flow not only across the
140 mitochondrial membrane, but across the plasma membrane as well (Jastroch et al., 2014). This could
141 be one key element explaining the negative impact of DNP on lifespan, but could potentially be solved
142 using *next generation uncouplers* (*e.g.* BAM15) being specific to the mitochondrial membrane

143 (Jastroch et al., 2014). Further studies investigating the molecular and physiological pathways by which
144 DNP shortens lifespan in zebra finches would be useful to enable targeted investigations of sublethal
145 deleterious effects in other animal models and potentially in humans. The present study should be a
146 potential warning signal for current illegal DNP users, and raise questions for scientists investigating
147 DNP use as a medicine.

148

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157

158 **Ethics**

159 Animal experimentation was conducted according to EU regulation (Directive 2010/63/EU) and was
160 approved by the ethical committee CREMEAS Strasbourg (#AL/02/02/01/13).

161

162 **Data availability**

163 Data used in this article is publicly available at: <https://figshare.com/s/6425205fd274d0c28bef>.

164

165 **Author contribution**

166 All authors contributed to study design, AS conducted the experiment with support from FC, PB and
167 SM. AS analyzed the data. AS & FC co-wrote the manuscript, with input from PB & SM.

168

169 **Competing interest statement:** the authors declare having no competing interests

170

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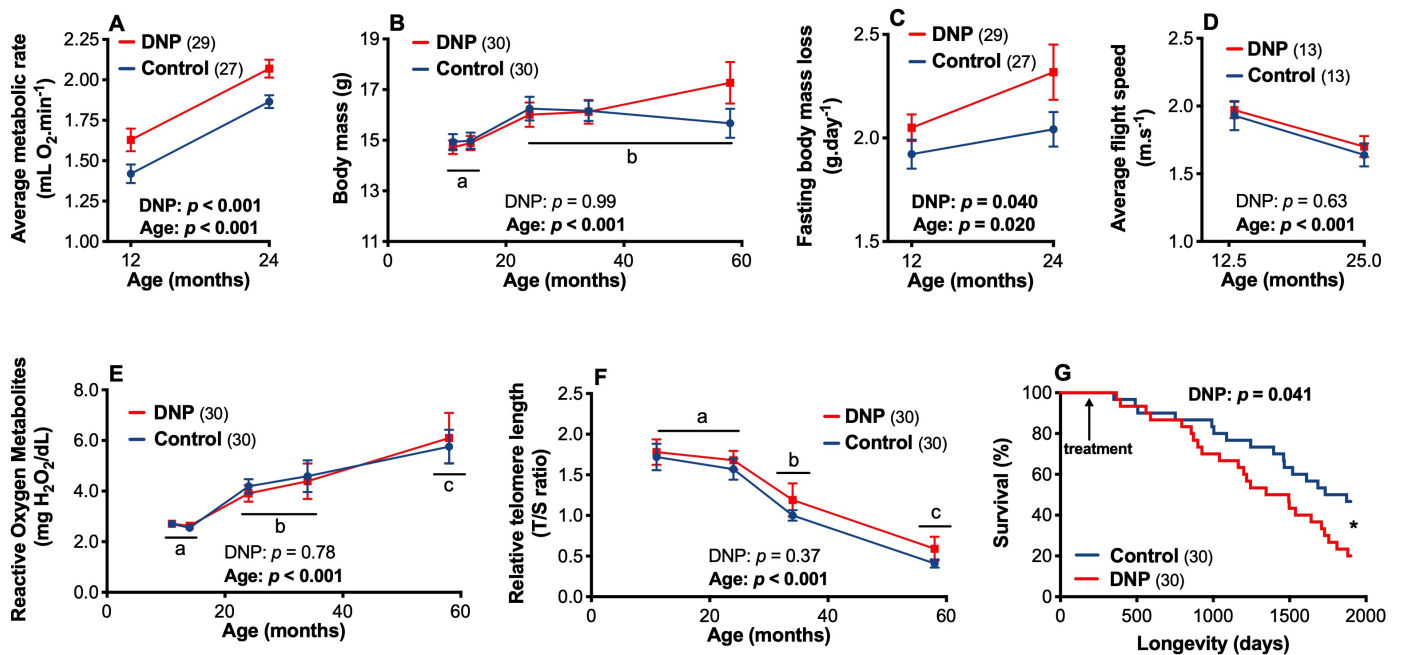
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235



249 **Fig. 1:** Zebra finches chronically treated with 2,4-Dinitrophenol (DNP) in their drinking water present:
 250 (A) a mild increase in **metabolic rate**, (B) no changes in the mild increase in **body mass** with age, (C) a
 251 higher **body mass loss during fasting**, (D) no change in the decrease of **locomotor (flight)**
 252 **performances** with age, (E) no change in the age-related increase in **oxidative damage**, or (F) the age-
 253 related **shortening of telomeres**. Yet, **DNP significantly reduces lifespan** (G). Control birds are
 254 indicated in blue and DNP birds in red, means are plotted \pm SE, *p*-values and N are presented within
 255 each panel and letters indicate significant differences according to sequential Bonferroni post-hoc
 256 tests for GEE models.

258 **Table 1: Summary of studies on the effects of chronic 2,4-Dinitrophenol (DNP) treatment, from yeast**
 259 **to humans.** () indicate observational data in humans based on early reports (~1930's) and poisoning
 260 incidents, ?: not tested, =: no significant change, ↓: decrease and ↑: increase.

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	Human	Mouse	Zebra finch	Frog tadpole	Drosophila	Yeast
DNP dose	(~1-12 mg.kg ⁻¹ .day ⁻¹)	~0.1 mg.kg ⁻¹ .day ⁻¹	~4 mg.kg ⁻¹ .day ⁻¹	1μmol.L ⁻¹ of water	0.1% in food	10nM
Body mass	(↓)	↓	=	=	?	?
Metabolic rate	(↑)	↑	↑	↑	=	↑
Oxidative stress	?	↓	=	↓	?	↓
Lifespan	?	↑	↓	?	↑	↑
Reference	Harris and Cocoran 1995	Caldeira da Silva et al. 2008	Stier et al. 2014; this study	Salin et al. 2012	Miquel et al. 1982	Barros et al. 2004

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Table 2: Results of GEE models testing the effects of age, DNP treatment and sex on (A) average metabolic rate, (B) body mass, (C) fasting body mass loss, (D) flight performance, (E) plasma ROMs levels and (F) blood cell relative telomere length

A. Metabolic rate (average VO₂)				
Fixed effects:	Estimate	Std. Error	Wald χ^2	p
Intercept	1.36	0.28		
Age (24mo)	0.38	0.07	43.38	< 0.001
Treatment (DNP)	0.21	0.07	12.77	< 0.001
Age*Treatment				(0.97)
Sex (F)	0.05	0.06	0.81	0.37
Body mass	0.04	0.02	5.69	0.017

B. Body mass				
Fixed effects:	Estimate	Std. Error	Wald χ^2	p
Intercept	16.09	0.59		
Age (11mo)	-1.44	0.43	38.79	< 0.001
Treatment (DNP)	-0.01	0.45	12.77	0.99
Age*Treatment				(0.70)
Sex (F)	0.33	0.45	0.81	0.46

C. Fasting body mass loss				
Fixed effects:	Estimate	Std. Error	Wald χ^2	p
Intercept	2.22	0.59		
Age (24mo)	0.19	0.08	5.44	0.020
Treatment (DNP)	0.19	0.09	4.20	0.040
Age*Treatment				(0.37)
Sex (F)	0.10	0.09	1.33	0.25

D. Flight performance				
Fixed effects:	Estimate	Std. Error	Wald χ^2	p
Intercept	2.22	0.59		
Age (25mo)	-0.28	0.05	28.82	< 0.001
Treatment (DNP)	0.05	0.10	4.20	0.63
Age*Treatment				(0.80)
Sex (F)	0.11	0.10	1.25	0.26

E. Plasma ROMs				
Fixed effects:	Estimate	Std. Error	Wald χ^2	p
Intercept	5.54	0.55		
Age (24mo)	1.38	0.55	74.86	< 0.001
Treatment (DNP)	-0.07	0.25	0.08	0.78
Age*Treatment				(0.89)
Sex (F)	0.81	0.25	10.77	0.001

F. Telomere length				
Fixed effects:	Estimate	Std. Error	Wald χ^2	p
Intercept	-0.25	0.19		
Age (34mo)	-0.66	0.12	89.09	< 0.001
Treatment (DNP)	0.09	0.10	0.81	0.37
Age*Treatment				(0.94)
Sex (F)	0.21	0.10	4.09	0.043
Initial telomere length	0.42	0.10	18.58	< 0.001

