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

Antoine Stier[1,2*], Pierre Bize[3], Sylvie Massemin[4] and François Criscuolo[4]

[1] Department of Biology, University of Turku, Turku, Finland

[2] Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow, UK

[3] School of Biological Sciences, University of Aberdeen, Aberdeen, UK

[4] Université de Strasbourg, CNRS, IPHC UMR 7178, F-67000 Strasbourg, France

[*]: corresponding author: antoine.stier@gmail.com / amstie@utu.fi

Antoine Stier https://orcid.org/0000-0002-5445-5524

Pierre Bize https://orcid.org/0000-0003-0454-2598

Sylvie Massemin https://orcid.org/0000-0002-4451-2812

François Criscuolo https://orcid.org/0000-0001-8997-8184

Keywords

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

Abstract

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

Introduction

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

DNP promotes body mass loss through a partial uncoupling of the oxidative phosphorylation (ATP production) system in mitochondria (Harris and Cocoran, 1995). When uncoupled, mitochondria are less efficient in converting energy and use more fuel to provide an equivalent amount of ATP (Brand, 2000). Concomitantly, mild mitochondrial uncoupling has the potential to reduce reactive oxygen species (ROS) production by the mitochondria, and thus to prevent oxidative stress and to extend lifespan according to the *uncoupling to survive hypothesis* (Brand, 2000). Experiments using DNP in various eukaryotic models (see Table 1) mostly support the *uncoupling to survive hypothesis*. However, DNP induces mitochondrial heat production, thereby making results from ectotherms (Table 1) potentially difficult to translate to endotherms, including humans. Additionally, the beneficial effects observed in mice (*i.e.* increased longevity, improved glucose-insulin-triglycerides plasma levels, decreased oxidative stress levels; Caldeira da Silva et al., 2008) might be associated with the anti-obesity effect of DNP in this species. Such beneficial effects might thus be absent in animal models not displaying age-related obesity or in non-obese humans. Despite its known toxicity (Harris and Cocoran, 1995), DNP has recently been granted an open Investigation New Drug (IND) approval by the FDA to begin clinical testing linked to its potential to prevent 'metabesity' (*i.e.* global comorbidities associated with the over-nutritional phenotype; Geisler, 2019). Therefore, it seems timely to evaluate the potential effects of chronic DNP treatment on ageing and lifespan using endotherm models not displaying age-related obesity.

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

Material & Methods

As explained in details in Stier et al. (2014), 60 captive zebra finches (32 females and 28 males) were randomly allocated to either a control group, or an experimental group treated with ~4 $mg.kg^{-1}.day^{-1}$ of DNP from 0.75 to 5.2 years of age. DNP treatment was administrated through the drinking water and did not result in any alteration of water intake (Stier et al., 2014). The DNP dose was chosen as the lowest dose eliciting an increase of whole-body metabolic rate (Stier et al., 2014). Individuals were followed longitudinally over the course of their life. Specifically, we measured body mass and collected blood samples at 11, 14, 24, 34 and 58 months of age. We estimated average metabolic rate at 12 and 24 months of age as overnight VO_2 (see Stier et al. (2014) for details), while also recording fasting body mass loss during the metabolic measurement (~10 hours) normalized to 24 hours (*i.e.* expressed in $g.day^{-1}$). We assessed vertical flight speed at 12.5 and 25 months of age following Reichert et al. (2015) as an indicator of physical performance and used its decline with age as a biomarker of ageing. We measured two biomarkers of ageing from blood samples. First, we measured oxidative damage as plasma reactive oxygen metabolites (ROMs) (see Stier et al. (2014) for details). Indeed, oxidative damage levels in the blood have been shown to increase with age, including in captive zebra finches (Marasco et al., 2017), and high levels of plasma ROMs have been associated with increased mortality risk in humans (Schöttker et al., 2015). Second, we measured relative telomere length of blood cells using qPCR (see Reichert et al. (2014) for details). Indeed, telomeres usually shorten with age, and short telomeres have been shown to predict increased mortality risk, including in captive zebra finches (Heidinger et al., 2012).

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

Results and Discussion

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

Ageing is a multifactorial process among which mitochondrial dysfunction, the accumulation of oxidative damage and the shortening of telomeres are suggested to play a role (López-Otín et al., 2013). DNP did not significantly prevent the age-related increase in oxidative damage levels over a period of *ca.* 4 years (Fig. 1E, Table 2E), confirming our previous results in early adulthood (Stier et al., 2014). Telomere length and shortening rate are thought to play a causal role in the ageing process (Muñoz-Lorente et al., 2019). Yet, we found no significant effect of chronic DNP exposure on telomere length or the age-related telomere shortening (Fig. 1F, Table 2F), suggesting no protective or detrimental effects of mild mitochondrial uncoupling on cellular ageing rate. Finally, our study highlights an overall detrimental effect of chronic DNP treatment on lifespan (median lifespan: DNP = 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 contradiction with previous experiments in other eukaryotic models (Table 1).

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

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

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

Acknowledgements

The authors wish to thank A. Hranitzky for support in bird maintenance, numerous students for their help in data collection, and N. Metcalfe, S. Dobson as well as S. Reichert for helpful comments on a previous draft.

Funding

AS was funded by a Marie Sklodowska-Curie Postdoctoral Fellowship (#658085) and a TCSM fellowship at the time of analyzing the results and writing the manuscript.

Ethics

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

Data availability

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

Author contribution

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

Competing interest statement: the authors declare having no competing interests

References

Ainsworth, N.P., Vargo, E.J., Petróczi, A., 2018. Being in control? A thematic content analysis of 14 in-depth interviews with 2,4-dinitrophenol users. International Journal of Drug Policy 52, 106–114. doi:10.1016/j.drugpo.2017.12.012

Barros, M.H., Bandy, B., Tahara, E.B., Kowaltowski, A.J., 2004. Higher Respiratory Activity Decreases Mitochondrial Reactive Oxygen Release and Increases Life Span in Saccharomyces cerevisiae. Journal of Biological Chemistry 279, 49883–49888. doi:10.1074/jbc.M408918200

Brand, M., 2000. Uncoupling to survive? The role of mitochondrial inefficiency in ageing. Experimental Gerontology 35, 811–820.

Briga, M., Jimeno, B., Verhulst, S., 2019. Coupling lifespan and aging? The age at onset of body mass decline associates positively with sex-specific lifespan but negatively with environment-specific lifespan. Experimental Gerontology 119, 111–119. doi:10.1016/j.exger.2019.01.030

Caldeira da Silva, C.C., Cerqueira, F.M., Barbosa, L.F., Medeiros, M.H.G., Kowaltowski, A.J., 2008. Mild mitochondrial uncoupling in mice affects energy metabolism, redox balance and longevity. Aging Cell 7, 552–560. doi:10.1111/j.1474-9726.2008.00407.x

Geisler, J., 2019. 2,4 Dinitrophenol as Medicine. Cells 8, 280–36. doi:10.3390/cells8030280

Grundlingh, J., Dargan, P.I., El-Zanfaly, M., Wood, D.M., 2011. 2,4-Dinitrophenol (DNP): A Weight Loss Agent with Significant Acute Toxicity and Risk of Death. J. Med. Toxicol. 7, 205–212. doi:10.1007/s13181-011-0162-6

Harris, M.O., Cocoran, J.J., 1995. Toxicological profile for dinitrophenols. Agency for Toxic Substances and Disease Registry.

Heidinger, B.J., Blount, J.D., Boner, W., Griffiths, K., Metcalfe, N.B., Monaghan, P., 2012. Telomere length in early life predicts lifespan. Proceedings of the National Academy of Sciences 109, 1743–1748. doi:10.1073/pnas.1113306109

Holmes, D., Flückiger, R., Austad, S., 2001. Comparative biology of aging in birds: an update. Experimental Gerontology 36, 869–883.

Holmes, D.J., Ottinger, M.A., 2003. Birds as long-lived animal models for the study of aging. Experimental Gerontology 38, 1365–1375. doi:10.1016/j.exger.2003.10.018

Hoxha, B., Petroczi, A., 2015. Playing with fire? Factors influencing risk willingness with the unlicensed fat burner drug 2,4-Dinitrophenol (DNP) in young adults. Public Health 129, 1519–1522. doi:10.1016/j.puhe.2015.03.013

Jastroch, M., Keipert, S., Perocchi, F., 2014. From explosives to physiological combustion: Next generation chemical uncouplers. Molecular Metabolism 3, 86–87. doi:10.1016/j.molmet.2014.01.003

Jeukendrup, A.E., Randell, R., 2011. Fat burners: nutrition supplements that increase fat metabolism. Obesity Reviews 12, 841–851. doi:10.1111/j.1467-789X.2011.00908.x

López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M., Kroemer, G., 2013. The hallmarks of aging. Cell 153, 1194–1217. doi:10.1016/j.cell.2013.05.039

Marasco, V., Stier, A., Boner, W., Griffiths, K., Heidinger, B., Monaghan, P., 2017. Environmental conditions can modulate the links among oxidative stress, age, and longevity. Mech Ageing Dev 164, 100–107. doi:10.1016/j.mad.2017.04.012

McVeigh, J., Germain, J., Van Hout, M.C., 2017. 2,4-Dinitrophenol, the inferno drug: a netnographic study of user experiences in the quest for leanness. Journal of Substance Use 22, 131–138. doi:10.3109/14659891.2016.1149238

Miquel, J., Fleming, J., Economos, A.C., 1982. Antioxidants, metabolic rate and aging in Drosophila. Arch Gerontol Geriatr 1, 159–165.

Muñoz-Lorente, M.A., Cano-Martin, A.C., Blasco, M.A., 2019. Mice with hyper-long telomeres show less metabolic aging and longer lifespans. Nat Commun 10, 1–14. doi:10.1038/s41467-019-12664-x

Reichert, S., Criscuolo, F., Zahn, S., Arrivé, M., Bize, P., Massemin, S., 2015. Immediate and delayed effects of growth conditions on ageing parameters in nestling zebra finches. J Exp Biol 218, 491–499. doi:10.1242/jeb.109942

Reichert, S., Stier, A., Zahn, S., Arrive, M., 2014. Increased brood size leads to persistent eroded telomeres. Frontiers in Ecology and Evolution 9. doi:10.3389/fevo.2014.00009/abstract

Salin, K., Luquet, E., Rey, B., Roussel, D., Voituron, Y., 2012. Alteration of mitochondrial efficiency affects oxidative balance, development and growth in frog (Rana temporaria) tadpoles. J Exp Biol 215, 863–869. doi:10.1242/jeb.062745

Schöttker, B., Brenner, H., Jansen, E.H., Gardiner, J., Peasey, A., Kubínová, R., Pająk, A., Topor-Madry, R., Tamosiunas, A., Saum, K.-U., Holleczek, B., Pikhart, H., Bobak, M., 2015. Evidence for the free radical/oxidative stress theory of ageing from the CHANCES consortium: a meta-analysis of individual participant data. BMC Med 13, 1315. doi:10.1186/s12916-015-0537-7

Stier, A., Bize, P., Roussel, D., Schull, Q., Massemin, S., Criscuolo, F., 2014. Mitochondrial uncoupling as a regulator of life-history trajectories in birds: an experimental study in the zebra finch. J Exp Biol 217, 3579–3589. doi:10.1242/jeb.103945

Figures

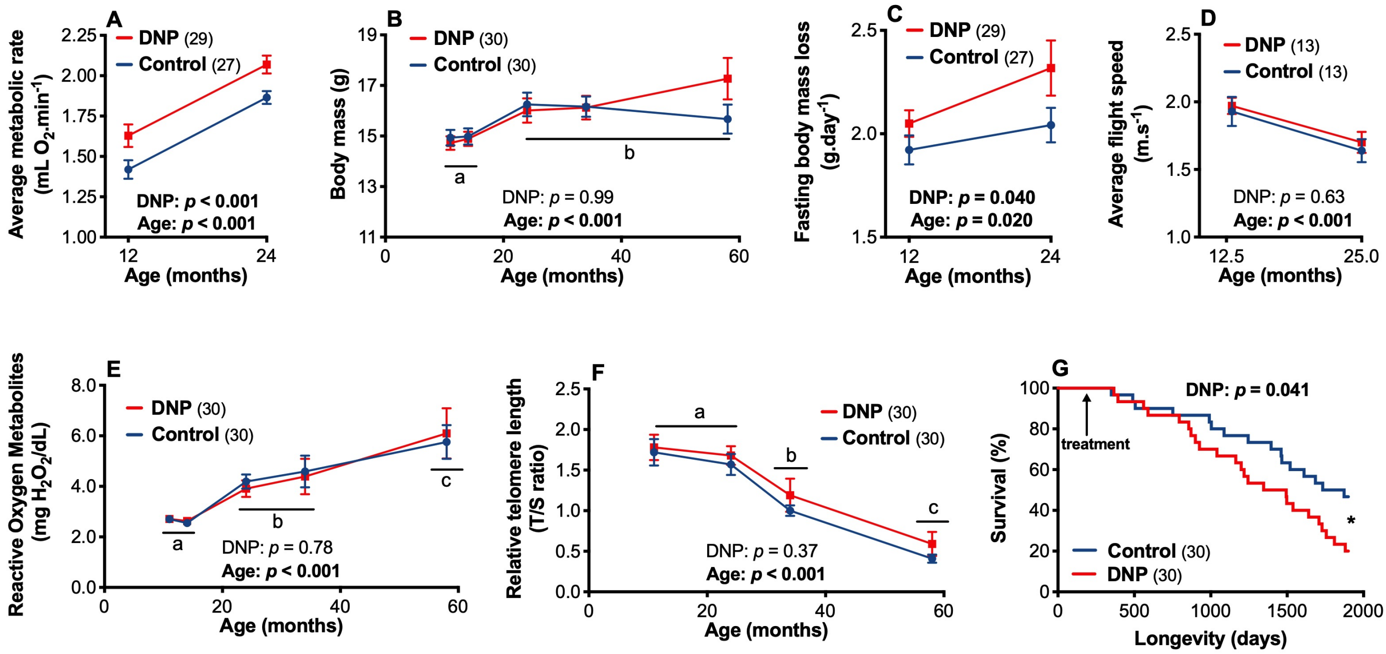

Fig. 1: Zebra finches chronically treated with 2,4-Dinitrophenol (DNP) in their drinking water present: (**A**) a mild increase in **metabolic rate**, (**B**) no changes in the mild increase in **body mas**s with age, (**C**) a higher **body mass loss during fasting**, (**D**) no change in the decrease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

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

	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

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 VO_2)

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**