PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2008) **275**, 1703–1708
doi:10.1098/rspb.2008.0357
Published online 29 April 2008

The impact of dietary restriction, intermittent feeding and compensatory growth on reproductive investment and lifespan in a short-lived fish

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While dietary restriction usually increases lifespan, an intermittent feeding regime, where periods of deprivation alternate with times when food is available, has been found to reduce lifespan in some studies but prolong it in others. We suggest that these disparities arise because in some situations lifespan is reduced by the costs of catch-up growth (following the deprivation) and reproductive investment, a factor that has rarely been measured in studies of lifespan. Using three-spined sticklebacks, we show for the first time that while animals subjected to an intermittent feeding regime can grow as large as continuously fed controls that receive the same total amount of food, and can maintain reproductive investment, they have a shorter lifespan. Furthermore, we show that this reduction in lifespan is linked to rapid skeletal growth rate and is due to an increase in the instantaneous risk of mortality rather than in the rate of senescence. By contrast, dietary restriction caused a reduction in reproductive investment in females but no corresponding increase in longevity. This suggests that in short-lived species where reproduction is size dependent, selection pressures may lead to an increase in intrinsic mortality risk when resources are diverted from somatic maintenance to both growth and reproductive investment.

Keywords: growth; fish; ageing; resource allocation

1. INTRODUCTION

While it is intuitively obvious that the lifespan of an animal will be affected by its diet, the degree (and even the direction) of the effect is not always predictable. It has long been known that in general, a diet that is supplied at less than the ad libitum amount can prolong both average and maximum lifespan, provided that it is above a lower threshold of malnutrition. This effect of dietary restriction has been observed across a diverse range of taxa, with similar results obtained whether or not the restriction is of the total diet or just specific components (e.g. calorie or protein restriction; Merry 1995; Masoro 2002, 2005; Piper *et al.* 2005). However, despite this generality and the long history of research on this phenomenon, the mechanism underlying it is still not clear and may indeed differ across different taxonomic groups (Anson *et al.* 2005; Masoro 2005; Sinclair 2005). A diet-induced change in lifespan can be due to either retarded or delayed ageing: in other words, a change in either the slope or the elevation of the mortality trajectory (Finch 1990; Merry 2005; Partridge *et al.* 2005). While a change in the slope of the trajectory is easily interpreted as a change in the rate of ageing (e.g. slowed or accelerated senescence), a change in the elevation is less intuitive: it indicates a change in the instantaneous risk of death rather than any change in the rate at which mortality increases with age (Mair *et al.* 2003).

The majority of experimental studies of the effects of dietary restriction on lifespan have used dietary regimes in which the food was supplied daily (albeit at a reduced rate). Nonetheless, a regime that alternated between periods of feeding and of starvation still found similar trends for lifespan extension, even though the total amount of food supplied was equal to that of ad libitum fed controls (i.e. the greater amount consumed on days when food was available, made up for the days when it was withheld; Anson *et al.* 2003). The cause of the lifespan extension noted in that study cannot therefore have been due to a reduction in nutrient intake, but is presumably a consequence of the periods of fasting. However, this is an intriguing result, since periods of food deprivation are commonly followed by compensatory ('catch-up') growth, which itself has been linked to a *reduction* in lifespan (Metcalfe & Monaghan 2001, 2003; Hales & Ozanne 2003).

The question remains of how these contradictory results can be reconciled. The disparity is not due to differences in study species, since small mammals have been used to show that periods of fasting followed by feeding can either extend lifespan (Goodrick *et al.* 1982; Sogawa & Kubo 2000; Anson *et al.* 2005) or reduce it (Ozanne & Hales 2004, 2005). Instead, it may arise due to differences in the exact nature of the protocol, for instance, the duration of the periods of fasting/re-feeding, the age and developmental stages of the animal and its life-history strategy. The majority of studies showing that intermittent feeding increases lifespan have used brief periods of food deprivation (Anson *et al.* 2005) that may not cause much reduction in growth, whereas the long-term deleterious effects of compensatory growth are strongest when the acceleration of growth is greatest (as happens when the deprivation either occurs early in growth or is prolonged, and/or the animal is

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Received 13 March 2008
Accepted 4 April 2008

approaching a life stage when Darwinian fitness is size dependent; Metcalfe & Monaghan 2001).

A further complication is that lifespan will be influenced by the relative investment of resources in body repair mechanisms, which, in turn, will depend on reproductive investment. Since resources are finite, the greater the resources invested in reproduction (whether this involves actual breeding or merely the development of mature gonads) the lesser that will be available for somatic maintenance. Therefore, some of the changes in lifespan associated with dietary restriction regimes may be due to the animal altering its investment in reproduction (e.g. by suppressing breeding as a response to adverse environmental conditions, and so being able to allocate proportionately more resources to maintenance; Shanley & Kirkwood 2000). However, studies of dietary effects on lifespan virtually never record reproductive investment, so making interpretation difficult.

In order to tease apart these confounding effects, we investigated the effects of intermittent feeding, compensatory growth and dietary restriction on both mortality trajectories and reproductive investment. Using experimental studies of a short-lived fish, the three-spined stickleback *Gasterosteus aculeatus*, we show that animals going through cycles of short-term deprivation followed by compensatory growth had a much shorter lifespan but the same investment in reproduction as those able to feed each day on the same total amount of food. By contrast, continuous dietary restriction has no effect on lifespan but a reduction in female reproductive effort.

2. MATERIAL AND METHODS

(a) *Husbandry protocol*

Fish were collected from the River Endrick, Stirlingshire, Scotland (OS grid ref NS 523 878) during November 2003 using dip nets and minnow traps. They were held at 9°C under an ambient photoperiod and allowed to acclimate in 100 l aquarium tanks for several months prior to the start of the experiment. All fish were starved for 24 hours before measuring to ensure evacuation of gut contents.

On 23 February 2004, the fish were starved for 24 hours to ensure evacuation of gut contents, and then weighed (to 0.001 g) and measured (to 1 mm). They were then randomly allocated to either a dietary restriction group (group DR: $n=40$; mean standard length ($\pm$s.e.) 33.8 ± 0.8 mm and mean weight 0.479 ± 0.004 g), control group (group CON: $n=32$; standard length 33.9 ± 0.9 mm and weight 0.488 ± 0.003 g) or an intermittent feeding group (group IF: $n=44$; standard length 33.5 ± 0.7 mm and weight 0.457 ± 0.003 g). It was predicted that fish were less likely to become sexually mature and breed in the DR and IF treatments, and so sample sizes per treatment were selected to compensate for this effect (so allowing analysis of the effects of these regimes on reproductive investment).

Fish were then housed in individual compartments ($10\times10\times20$ cm) in a flow-through glass-sided aquarium (flow rate 12 l min^{-1} and temperature 11 ± 1°C). The floor of each compartment was covered with pea gravel and each compartment was supplied with a plastic plant; the end walls of each compartment were of mesh so that fish could see their neighbours. Bloodworms were used as food. Based on previous studies of stickleback energy budgets (Allen & Wootton 1982), the CON and DR groups were fed daily with 10 and 2% of their body weight in bloodworms (larval *Chironomus* spp.), respectively. This allowed unrestricted growth in the CON group, and very limited growth in the DR group. Fish in the IF group received the same total amount of food as those in the CON group over a 14-day period. However, they alternated between periods of fasting and feeding, being starved for 5 consecutive days and then fed on approximately 15% of their body weight for 9 days. Three-spined sticklebacks on this kind of cyclical regime undergo repeated compensatory growth fuelled by hyperphagia and increased growth efficiency during each period when food is available, such that they can maintain similar growth rates to controls (Ali & Wootton 2001; Wu *et al.* 2003). The feeding schedules were authorized by the University's Ethical Review Committee and by the UK Home Office under Project licence no. 60/2894.

All fish were weighed and measured at two-weekly intervals (at the end of each period of starvation for the IF fish) until the end of the experiment, being starved for 24 hours before each measurement. Specific growth rate was calculated as $100(\ln M_{\mathrm{f}}-\ln M_{\mathrm{i}})/t$ for weight and length, where M_{i} and M_{f} refer to the initial and final measurements, respectively, and t is the time interval in days. Each fish was maintained on the same feeding regime until its death. Throughout the experiment, fish were checked for signs of maturation (females: swelling of the abdomen indicating a fish had become gravid and males: development of a blue iris and/or red throat). Data were collected until 16 August 2005, when only four fish remained alive. Typically, fish from the study population breed for just a single season (Pike *et al.* 2007). While a small number of fish survived until their second breeding season, none of the females produced eggs in this second year and the males did not produce full breeding coloration, therefore, only reproductive data for the first breeding season are included here. For the purposes of statistical analysis, a nominal birth date (1 May 2003) was assumed for all fish. The longevity data were analysed using Cox's regression analysis with the four fish still alive being included as censored cases.

(b) *Reproductive investment*

A protocol adapted from Frischknecht (1993) was used to record throat coloration of all males that had developed a blue (i.e. reproductive) iris colour. Each male was netted from its compartment and temporarily placed in a thin water-filled chamber that allowed the left lateral surface of the fish to be photographed against an opaque neutral background and alongside a colour chart (Jessop's colour and monochrome separation guide) and small piece of laminated squared paper. Photographs were taken using a tripod-mounted Canon Expedia 3040 digital camera (shutter speed 1/400 s, f 3.2). Illumination was provided from two full spectrum daylight bulbs angled at 45° to the tank; the relative position of both lamps and camera was kept constant in all photographic sessions. Previous work (Braithwaite & Barber 2000) has shown that a single lateral photograph of the males' breeding colours gives similar results to photographing both the lateral and ventral sides; therefore, only one photograph was taken in order to minimize stress to the animal and so prevent stress-mediated colour bleaching. The complete process of moving the fish from its home compartment to the experimental tank and taking the photo took less than 60 s. Coloration measurements were made at five time points from soon after the start of the breeding season, until all males had lost

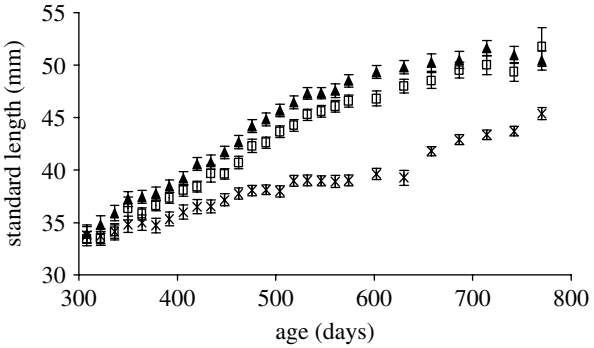

Figure 1. Mean standard length (mm; ±s.e.) of sticklebacks in relation to diet treatment (intermittent feeding, open squares; dietary restriction, crosses and control, triangles).

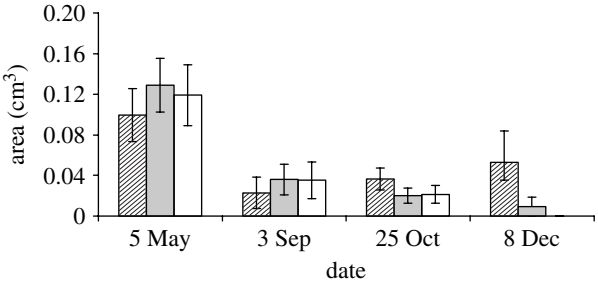

Figure 2. Mean size (cm^3; ±s.e.) of the area of red nuptial coloration in males sticklebacks from the three dietary regimes in relation to time of year (intermittent feeding, hatched bars; dietary restriction, filled bars and control, open bars). See text for statistical analysis.

their red coloration. This produced data for 5 May, 8 July, 3 September, 25 October and 8 December; however, data from 8 July were lost due to a computer malfunction and therefore could not be included in the analysis.

Image analysis was performed using ImageJ software. The red area of the ventral side of the male was selected as described by Barber *et al.* (2000), and its area measured using the measure function. A score was obtained for redness, greenness and blueness of both the selected patch on the fish and the red area of the colour chart using the RGB Measure plugin. To equalize any light or tone differences between the pictures, all values obtained for the coloration of the fish were standardized by dividing them by the value of the colour levels obtained for the standard in each picture (I. C. Cuthill 2004, personal communication), e.g. red_{STD}(standardized colour value for redness) = (mean intensity of red brightness of selected area on fish)/(mean intensity of brightness of selected area on red colour standard). This was repeated for the blue and green channels. Red intensity (R) was calculated as $red_{STD}/(red_{STD} + green_{STD} + blue_{STD})$ where R represents the red component of the selected area. High values of R indicate that a high proportion of the total image brightness is made up of the red channel (Barber *et al.* 2000).

Females were observed daily and any that appeared gravid (i.e. had a grossly distended abdomen) were removed from their tanks and stripped of eggs under light anaesthetic (benzocaine) following the protocol of Ali & Wootton (1999). All eggs were collected in a Petri dish. The standard length and pre- and post-stripping weights of the female were recorded, since fecundity is related to body size (Wootton 1973). The female was then returned to her tank. The total number of eggs was counted, and the diameter of either 50 eggs or the total number of eggs (whichever was lesser) was measured to 0.1 mm using a dissecting microscope and graticule. The condition factor of all females was calculated as the residuals of a regression of body mass against standard length at the peak of the breeding season.

3. RESULTS

(a) *Growth rates*

At the beginning of the experiment, there were no differences in the mean standard length or weight of fish in the three treatments (ANOVA, standard length: $F_{2,115} = 0.10$, $p = 0.91$; weight: $F_{2,115} = 0.20$, $p = 0.82$). Sizes in both weight and length began to diverge within two months of the feeding treatments (figure 1; weight data not shown but show similar trends). Repeated measures ANOVA on standard length up until 580 days (i.e. before mortality caused a significant loss of sample size) showed effects of date ($F_{19,67} = 52.14$, $p < 0.001$), treatment ($F_{2,85} = 17.87$, $p < 0.001$) and date by treatment ($F_{38,136} = 3.47$, $p < 0.001$). Contrasts showed that IF fish grew as quickly as CON fish ($p = 0.09$), whereas DR fish were slower ($p < 0.001$).

(b) *Reproductive investment*

The area of the male red nuptial coloration at the peak of the breeding season (5 May) was positively related to body size (ANCOVA, effect of standard length: $F_{1,43} = 13.08$, $p = 0.001$) but did not differ between treatments ($F_{2,43} = 0.11$, $p = 0.90$). The extent of this red area subsequently declined through to late autumn but the changes were similar in the three treatment groups (repeated measures ANOVA, effect of time: $F_{3,31} = 6.44$, $p = 0.002$; effect of treatment: $F_{2,33} = 0.08$, $p = 0.93$; figure 2). The intensity of the redness on 5 May was independent of both body size (ANCOVA, effect of standard length: $F_{1,43} = 2.20$, $p = 0.15$) and treatment ($F_{2,43} = 0.31$, $p = 0.74$). The intensity also declined over time, again independently of dietary treatment (repeated measures ANOVA, effect of time: $F_{3,99} = 29.92$, $p < 0.001$; effect of treatment: $F_{1,33} = 0.50$, $p = 0.61$).

A smaller proportion of females spawned in the DR group (3/22) than in either of the other two groups (IF: 10/20 and CON: 12/17), but this was primarily an effect of the dietary regimes on body size: logistic regression showed that the probability of spawning was independent of treatment (Wald statistic = 4.80, d.f. = 2, $p = 0.09$) after controlling for standard length at the peak of the breeding season (Wald statistic = 4.49, d.f. = 1, $p = 0.034$). However, there was a significant effect of treatment on the total number of clutches produced by females (ANCOVA, with standard length as a covariate; effect of treatment: $F_{2,54} = 3.64$, $p = 0.032$; standard length: $F_{1,54} = 1.72$, $p = 0.20$; length by treatment interaction not significant ($F_{2,52} = 1.97$, $p = 0.15$) so removed from model). *Post hoc* tests showed that DR females produced significantly fewer clutches (mean ± s.e. = 0.14 ± 0.08; range 0–1; $n = 21$) than CON (1.12 ± 0.26; range 0–4; $n = 17$) or IF females (0.80 ± 0.23; range 0–3; $n = 20$), which did not differ in clutch production (DR versus IF: $p = 0.005$; DR versus CON: $p = 0.005$; CON versus IF: $p = 0.94$).

Considering only females that spawned, there was no treatment difference in condition factor, standard length or weight at the time of becoming gravid with the first clutch (ANOVAs, $p > 0.58$). Among these females, the

Table 1. Results of Cox's regression analysis on lifespan of sticklebacks, showing the independent significant effects of diet treatment and growth rate at start of diet manipulation (24 February–8 March 2004). (Overall significance of model: $\chi^2_4 = 28.82$, $p < 0.001$. Non-significant candidate variables were dropped from the model (see text).)

	Wald statistic	d.f.	p	exp(B)
treatment	23.86	2	<0.001	
growth rate in weight	5.24	1	0.022	0.813
growth rate in length	4.43	1	0.035	1.795

number of eggs produced per clutch was related to the fish's standard length (ANCOVA based on log–log transformations, $F_{1,19} = 7.78$, $p = 0.012$) but was unaffected by treatment ($F_{2,19} = 1.51$, $p = 0.25$) and there was no length by treatment interaction ($F_{2,17} = 0.04$, $p = 0.96$); mean ln(nos.) of eggs per clutch (±s.e.; adjusted to a common standard length of 37.9 mm) were 3.67±0.11, 3.81±0.06 and 3.89±0.06 for DR, IF and CON females, respectively. The mean size (diameter) of a female's eggs was unrelated to her treatment group ($F_{2,21} = 0.29$, $p = 0.75$) or standard length at the peak of the breeding season ($F_{1,21} = 0.03$, $p = 0.86$; length by treatment $F_{2,19} = 0.01$, $p = 0.99$); mean values were 1.27±0.03, 1.28±0.06 and 1.27±0.06 mm for DR, IF and CON females, respectively.

(c) *Lifespan*

Mortality trajectories were analysed using Cox's regression analysis, with treatment and sex as main effects, and standard length/weight at the beginning of the experiment (24 February 2004) and initial growth rate at that time as covariates. Later measurements of size or growth rate could not be used as some fish had died by that point. A backwards stepwise approach was used, with non-significant terms being sequentially dropped (based on likelihood ratios). Sex, initial length and weight were dropped as being non-significant ($p > 0.24$), producing a final model containing the terms treatment, growth rate in length and growth rate in weight (table 1). IF fish died significantly sooner than either CON (figure 3*a*; Wald statistic = 14.57, d.f. = 1, $p < 0.001$) or DR fish (Wald = 20.92, d.f. = 1, $p < 0.001$). The median lifespan of IF fish was 582 days, in contrast to 740 days for CON and 746 days for DR fish, which did not differ in longevity (Wald = 0.36, d.f. = 1, $p = 0.55$). There were equivalent differences in the lifespan at which 90% of the fish in each treatment had died (IF = 740 days, CON = 816 days and DR = 820 days). Growth rates in length and weight had opposing effects: faster growth in body length was associated with a reduction in lifespan (the hazard rate increasing by 79.5% for every unit increase in percentage gain in length per day; table 1), whereas faster weight gain was associated with increased longevity (hazard rates declining by 28.7% per unit increase in percentage of gain in weight per day).

A reduced lifespan can either be due to an increase in the slope of the mortality trajectory (indicative of an increased rate of ageing) or an increase in the acute risk of death (i.e. a change in the elevation of the mortality trajectory, rather than its slope). Therefore, we plotted the

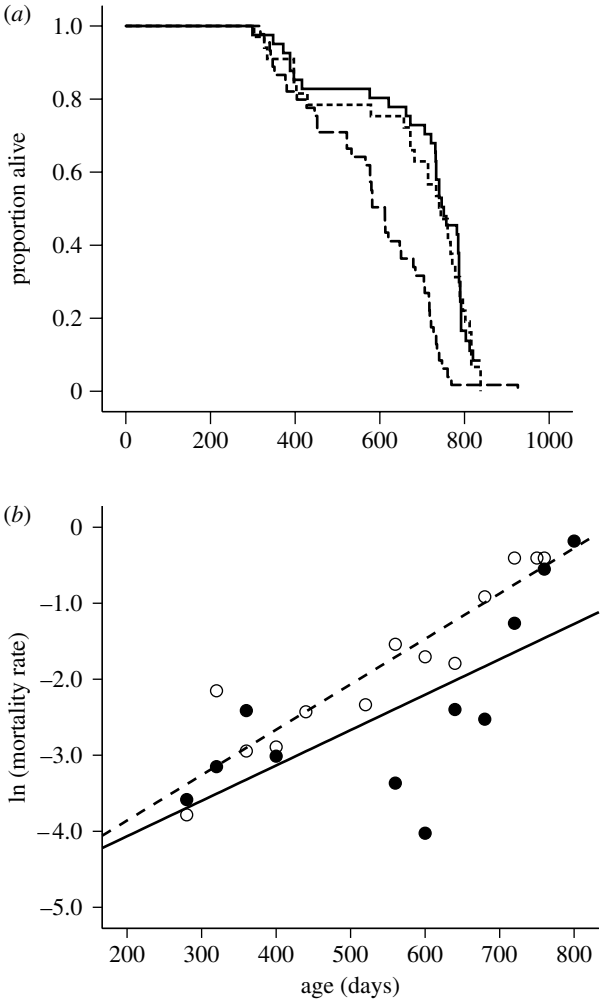

Figure 3. (*a*) Survival curves of sticklebacks in relation to diet treatment (intermittent feeding, dashed line; dietary restriction, solid line; control, dotted line). (*b*) Trajectories of mortality rate against age (intermittent feeding treatment, open circles and dashed regression line; control and dietary restriction treatments combined, open circles and solid regression line). See text for statistical analysis.

graphs of mortality rates against age for the different treatment groups. Since the survival curves for the CON and DR fish were so similar (figure 3*a*), the data for these two groups were combined to increase the resolution of the plots. Mortality rate was calculated as ln(fish dying per 40-day time interval, expressed as a proportion of those alive at the start of the interval); the analysis was curtailed when only four survivors were left. The mortality rate increases with age in all fish (figure 3*b*; ANCOVA $F_{1,21} = 19.24$, $p < 0.001$). However, while the slope of the mortality trajectory for IF fish did not differ from that of fish in the other two treatments ($F_{1,20} = 0.60$, $p = 0.45$), there was a significant difference in elevation ($F_{1,21} = 5.41$, $p = 0.03$), indicating that the dietary manipulation had changed the acute risk of death rather than the rate of ageing.

4. DISCUSSION

Sticklebacks on the intermittent feeding regime grew at the same rate as fish in the control group, which were receiving the same total amount of food but feeding every day. This ability to compensate for periods of deprivation has previously been documented in sticklebacks (Ali & Wootton 2001) and has been attributed to an increase in

gross growth efficiency during the repeated phases of compensatory growth (Wu *et al.* 2003). The IF fish also matched the controls for their investment in reproduction: males maintained a similar degree of red coloration, while females produced the same number and size of clutches, and the same egg size. However, there was a marked difference in both average and maximum lifespan, with IF fish of both sexes dying younger than either control fish or those on dietary restriction.

This shortening of lifespan may have been due to a reduced investment in somatic repair: the greater growth efficiency during the periods of growth acceleration when food was available (Wu *et al.* 2003) may have been achieved by a reduction in protein maintenance (Morgan *et al.* 2000) and hence faster accumulation of cellular damage and senescence (Jennings *et al.* 2000). Indeed, recent dietary manipulations in sticklebacks have shown that individuals that are less able to invest in defence against oxidative stress incur more oxidative damage and have a shorter lifespan (Pike *et al.* 2007). Rapid growth may be especially costly in terms of oxidative stress, not only because the greater metabolic activity that it entails generates more reactive oxygen species but also because resources are diverted into anabolism and away from repairing oxidative damage to proteins. It would appear that rapid growth in length is more costly than accumulation of weight, since (after controlling for diet treatment effects) individuals that were growing fastest in length (but not weight) had shorter lives.

One seemingly anomalous result in this study is that, in contrast to the majority of studies on diverse taxa (Merry 1995; Masoro 2002, 2005), there was no trend for the animals on a dietary restriction regimen to live longer than controls. This may be because in studies that find a positive effect of dietary restriction on lifespan, the subjects are generally in a non-reproductive state and are not approaching a time point when fitness is closely correlated with body size; the limited resources that are available can therefore be channelled into somatic maintenance without any conflict with reproduction or growth. This allows a greater investment in protein repair despite the reduction in resource intake (Dubey *et al.* 1996; Forster *et al.* 2000). By contrast, the sticklebacks in this study normally only breed over a single season, and would be under strong selection to reach as large a size as possible by the time of breeding (since both fecundity and male reproductive success are positively related to body size; Wootton 1973). Resource allocation may, therefore, have been shifted away from somatic defence and repair mechanisms (Poizat *et al.* 1999). This may also explain why intermittent feeding caused decrease lifespan in this study whereas previously it has been found to increase the lifespan in rodents (Goodrick *et al.* 1982; Sogawa & Kubo 2000; Anson *et al.* 2003)—possibly because in those studies the animals were neither breeding nor under a selection pressure to grow fast, and so were avoiding the generation of high levels of reactive oxygen species and could also divert resources to somatic repair.

The analysis of growth trajectories showed that the reduction in lifespan of IF fish was due to an increase in the short-term risk of death rather than an increase in the gradual rate of senescence; the trajectories were not strictly linear (due to a decrease in mortality rates in CON and DR fish after the breeding season) but nonetheless showed clear differences in elevation. This suggests that the IF fish were not ageing faster, but were more likely to die when in a given physiological state. This would fit with the concept that cellular damage accumulates as a result of an imbalance between the rate of damage occurring and the investment in repair: CON and DR animals may have been able to repair damage to proteins (Dubey *et al.* 1996; Forster *et al.* 2000) whereas the same damage in IF fish may have gone unrepaired, leading to a greater likelihood of death. The greatest difference in survival occurred during the period lasting from the middle of the first breeding season to the late autumn; while mortality rates of CON and DR fish were minimal over this time, those of IF fish continued to rise. While it is unlikely that this higher mortality was due to increased reproductive investment (since this did not differ between IF and CON animals), it may be the case that the normal diversion of resources into reproduction caused greater damage in IF fish due to impaired defence or repair mechanisms (Poizat *et al.* 1999; Pike *et al.* 2007).

The cellular processes that cause death are extremely difficult to elucidate, but this study provides clear evidence that the pattern of food intake and consequent growth trajectory can have major implications on life histories: fish in the CON and IF treatments consumed the same total amount of food and grew to approximately the same size, but the intermittent nature of feeding and consequent saltatory growth pattern of the IF fish caused a significant increase in their risk of death, leading to a shorter mean and maximum lifespan. Future studies should concentrate on the mechanistic links between growth rate and rates of cellular damage, repair and senescence.

We thank Tom Pike and Innes Cuthill for advice on measuring fish coloration, John Laurie for fish husbandry and two referees for their helpful comments. C.L.W.I. was funded by a PhD Studentship from NERC.

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