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Rest-activity circadian rhythms in aged *Nothobranchius korthausae*. The effects of melatonin

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ABSTRACT

Adult (48-week-old) and senescent (72-week-old) individually-kept *Nothobranchius korthausae* were used as experimental subjects to characterise circadian system (CS) function and age-related changes in senescent fish. This species was specifically chosen because it has already shown potential for use as a model system in gerontological studies. The rest-activity rhythm (RAR) in fish can be easily monitored and used to characterise the state of the CS, and it has also been proposed as a reliable model to study sleep-like periods in fish. As they aged, *N. korthausae* experienced a significant decrease in total daily activity and a progressive impairment of the RAR, accompanied by changes in the regularity, fragmentation and amplitude of the rhythm. The ability of the CS to oscillate autonomously when the two main synchronizers, photoperiod and feeding time, were absent (continuous darkness and random feeding), was also impaired with age, as the capacity to re-synchronise to the light–dark (LD) cycle declined. Melatonin treatment improved the regularity, fragmentation and amplitude of the RAR in senescent fish, and it also improved sleep efficiency. In conclusion, *N. korthausae* represents a viable model for studying the aging of the circadian system and the restorative effect of chronobiotic substances, such as melatonin.

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1. Introduction

Aging is a ubiquitous process, characterised by an increase in cell oxidative stress and the progressive accumulation of deleterious changes in macromolecules (Harman, 2006). Aging also involves modifications in intercellular communications, required for coordinated responses to external and internal events which could be related with an age-dependent failure in the integrating systems of an organism, including the circadian system (CS) (Weinert, 2000). CS structure and function have been shown to undergo several changes throughout the life-cycle of an organism, particularly during ontogeny and senescence (Turek et al., 1995; Weinert, 2005; Weinert and Schuh, 1988). Damage to any element of the CS will be reflected in overt rhythms, such as daily variations in locomotor activity, known as the rest-activity rhythm (RAR), this rhythm constitutes a potential tool for monitoring the changes in the functional organisation of animals as they age (Mailloux et al., 1999). RAR has been shown to suffer from age-related impairment in humans and mammals (Kondratov, 2007; Van Someren et al., 1999) and also in fish (Zhdanova et al., 2008), already accepted as vertebrate models for gerontological research (Gerhard, 2007). Some species of fish, mainly zebrafish and some species from the genus *Nothobranchius* have been shown to be good models for studies on aging, this being important due to the traditional lack of vertebrate models for these types of studies. They have a short life-cycle, can be easily maintained, and reproduce well under laboratory conditions; they also show gradual senescence, characterised by different biomarkers based on phenotypic, biochemical, behavioural and cognitive factors (Gerhard et al., 2002; Hartmann et al., 2009; Hsu and Chiu, 2009; Hsu et al., 2008; Terzibasi et al., 2009; Valenzano et al., 2006).

The organisation of the CS in fish shows some differences from that in mammals. The suprachiasmatic nucleus, the circadian master clock in mammals, has not yet been shown to be a central pacemaker in fish. On the contrary, the pineal gland has been suggested to play a central role in fish circadian rhythmicity (Zhdanova and Reebs, 2006). The pineal gland rhythmically releases melatonin and synchronises the animal functions by acting on a complex network of central and peripheral clocks that are closely coordinated in order to maintain physiological integrity (Peirson et al., 2009; Whitmore et al., 1998, 2000; Zhdanova and Reebs, 2006). Some studies have analysed RAR age-related changes in fish, mainly using zebrafish as a model (Zhdanova et al., 2008). These changes include a decrease in overall daily activity, the loss of a rhythmic pattern and a reduction in rest time associated with an impaired melatonin rhythm. In a recent publication, we studied the effects of aging on the RAR of *Nothobranchius korthausae*, a species with an average lifespan of 18 months, quite shorter than that of zebrafish (up to 6 years). We found a loss of amplitude, regularity and increased rhythm fragmentation with age in fish groups (Lucas-Sánchez et al., 2011).

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In the evolution of vertebrates, one of the processes associated with the RAR that shows age-related changes is sleep. Sleep is a resting state that has been observed in all vertebrates, and it is regulated by the interaction of two other processes: a circadian modulation controlled by the CS and a homeostatic regulation (Bes et al., 2009). Even though the role of sleep is not entirely clear, it is known that it is associated with several important functions, such as learning, memory, homeostatic restoration and tissue repair (McNamara et al., 2009). The negative impact of sleep loss or sleep disorders on health makes it an interesting topic to consider along with the general state of the RAR in studies of functional deterioration under high-stress situations and with aging (Riemersma et al., 2004; Van Cauter et al., 2008; Walker, 2008). A common species used for sleep studies and with a real potential for this purpose is the zebrafish (Wang et al., 2011; Zhdanova, 2011), although other species of teleost fish also proposed as aging models could be useful for this purpose. Zebrafish displays physiological and behavioural mechanisms that have been described as sleep-like states (Wang et al., 2011; Yokogawa et al., 2007; Zhdanova, 2011). These states can be modulated by sleep promoters/inhibitors and drugs, and that they can be analysed by characterising the animal's behaviour.

The aim of the present work was to analyse the changes in the RAR of senescent fish of genus *Nothobranchius* in order to characterise the state of the aged CS and also to test the RAR potential usefulness for evaluating sleep-like states and the effects of exogenous melatonin. *N. korthausae* was specifically chosen because it has shown potential for use as a model system in gerontological studies. Its intermediate lifespan, significantly longer than that of other species of the genus, such as *Nothobranchius furzeri* or *Nothobranchius rachovii*, is still quite shorter than that of the zebrafish and potentially allows for long-term studies to be performed at various stages in the aging process.

2. Materials and methods

2.1. Animal housing and data acquisition

N. korthausae Meiken 1973 (Actinopterygii, Cyprinodontiformes, Nothobranchiidae) from the Mafia Island population (TAN 02–5), belonging to a resident population established in the Fish Chronobiology Laboratory at the University of Murcia, were used. The maximum lifespan under laboratory conditions of this species has been established as 81 weeks. Fish were kept under constant temperature (26 ± 2 °C) in 70-L tanks equipped with a recirculating fresh water system (4 L/h flow) and biological and mechanical filtration. The water parameters were controlled to conform to the following values: water hardness < 6 dKH, NO_3^- < 0.1 mg/L, NO_2^- < 0.1 mg/L, NH_3 < 0.5 mg/L and pH = 7.4. The photoperiod was set at 12L:12D (lights on at 08:00 h, ZT_0). Light intensity at the surface of the water during the photophase was 200 lx. More information about fish housing and maintenance has already been provided in a previous publication (Lucas-Sánchez et al., 2011).

For the present experiments, fish with an average body weight of 1.04 ± 0.16 g and body length of 40 ± 2 mm were individually kept in 12-L tanks (dimensions: 20 × 30 × 20 cm) equipped with two photoelectric sensors (model E3S-AD62, Omron Corporation, Kyoto, Japan), one positioned 5 cm below the water surface, and the other placed 5 cm above the bottom of the tank. When the fish swam in front of the sensors attached to the glass aquariums, the interruption of the infrared light beam was recorded as one count. The number of counts was collected every 10 min, using a data acquisition system (Electronic Services of the University of Murcia, Spain). Total activity was calculated as the sum of the data from the two photoelectric sensors in each tank.

2.2. Experimental procedures

2.2.1. Rest-activity rhythms in the senescent stage

In order to characterise RARs and their changes with aging in *N. korthausae*, the locomotor activity of each six adult (48-week-old) and senescent (72-week-old) male fish was monitored. Fish were automatically fed dried worms (PRODAC, Padova, Italy) once a day at 14:00 h (ZT_6). The fish were first placed for 15 days under a photoperiod of 12L:12D (Phase 1) as a control, and then (Phase 2) subjected to continuous darkness (DD) and automatic random feeding (the feeding time was changed day by day) for another 15 days to test for endogenous circadian rhythmicity in both age groups. They were then allowed to recover (Phase 3, 15 days) under the same conditions as in Phase 1 to check the ability of adult and senescent fish to re-synchronise to the initial conditions. Both an initial (Phase 3a, 7 days) and a final recovery period (Phase 3b, 7 days) were identified within Phase 3.

2.2.2. Effects of melatonin treatment on senescent fish RAR

Ten 72-week-old male fish were used to test for the effects of melatonin treatment on RAR in senescent *N. korthausae*. Fish were manually fed a dried worm diet twice a day, at 10:00 h (ZT_2) and 19:00 h (ZT_{11}), and were kept under a 12L:12D photoperiod. A solution of melatonin (0.5 g/100 ml, SIGMA, M-5250, Milwaukee, WI, USA) in 99.9% ethanol (J.T. Baker, 02422460012, Deventer, The Netherlands) was prepared, and 8.92 ml of this solution was sprayed on 20 g of dried worm diet prior to feeding, basically as described by Amano et al. (2004). The melatonin content was 2.2 mg/g-dried worms. The control worm diet was sprayed only with ethanol. During the control period (Phase 1, 15 days), fish were fed the control worm diet at ZT_2 and ZT_{11}. This phase was used to characterise the animals' RAR status. During melatonin treatment (Phase 2, 15 days), fish were fed manually the control worm diet at ZT_2 and melatonin-supplemented worm diet at ZT_{11} for a total dose of 5 mg melatonin/kg-BW/day, observing that it was ingested. Finally, during Phase 3 (20 days), fish were fed as in Phase 1 to detect potential post-treatment effects.

The experimental procedure abides by current Spanish law regarding animal experiments, and the experimental protocol was approved by the Bioethics Committee of the University of Murcia.

2.3. RAR analysis

Rest-activity rhythms were analysed by processing locomotor activity records according to the two different methods as described below.

2.3.1. General overview of RAR

A software specifically designed for time series analyses (El Temps, A. Díez-Noguera, Universitat de Barcelona, 1999) was used to compute: a) *Total activity* (counts/day); b) *Percentage of nocturnal activity*; c) *Mesor* (counts/10 min); d) Acrophase regularity according to the *Rayleigh test* (*r*); e) the *Circadian/Ultradian ratio*, calculated as the relationship between the power (percentage of variability accounted for) of the first harmonic (fit to a 24-hour sinusoidal wave) and the sum of all harmonics analysed (up to the 6th harmonic) corresponding to sinusoidal waves of less than 24 h; and f) the percentage of variance accounted for by the highest peak in the periodogram (*Periodogram %V*), which is used as an indicator of the robustness of the locomotor activity rhythm.

2.3.2. Non-parametric indices

Non-parametric indices were calculated as described by Van Someren et al. (1999), and they included: a) *Intradaily variability* (*IV*), which oscillates between 0 and 2, depending on the endogenous character of the circadian rhythm, and is a measure of rhythm fragmentation; b) *Interdaily stability* (*IS*), which quantifies rhythm stability over different days and is more dependent on external factors; and c) *Relative amplitude* (*RA*), which results from internal and external influences. IS and RA oscillate between 0 and 1. Information provided by the above IV, IS and RA was integrated in a single value, the *Circadian Function Index* (*CFI*) (Ortiz-Tudela et al., 2010). For this purpose, IV values were first inverted and normalised between 0 and 1, and then averaged

with the other two parameters to compute the CFI. This index conveys information on the general functioning of the circadian system and oscillates between 0 (absence of circadian rhythmicity) and 1 (a robust circadian rhythm).

2.4. Sleep quality

According to the behavioural criteria previously described in the literature (Campbell and Tobler, 1984), *N. korthausae* shows sleep-like states (hereinafter, sleep) that can be defined as short periods of inactivity during the nocturnal phase, floating with the head slightly upward generally at the surface of the tank, and with no pectoral fin movements. In our experiments, sleep was considered to occur when locomotor activity (measured with photoelectric sensors) dropped below the 33rd percentile of total nocturnal activity. To characterise sleep quality in this species, the following indices were calculated: a) number of *Sleep interruptions* during the rest period; b) *Interruption duration*; c) *Sleep latency*, as the time spent between lights off and the first period of inactivity (considering inactivity as 50 min below the 33rd percentile of total nocturnal activity); d) *Sleep offset*, as the time in anticipation of lights on, which was considered as the awakening time (5 consecutive increases in locomotor activity); e) *Rest time*, as the time elapsed between the first period of inactivity (defined by sleep latency) and the first period of activity in the next light period (defined by sleep offset); and f) *Sleep efficiency*, calculated as (*Rest time* − *Rest interruptions* × *Interruption duration*) / night duration (Supplementary Fig. 1).

2.5. Statistical analysis

All data are presented as mean ± SEM. In Experiment 1, statistical comparisons between adult and senescent *N. korthausae* for Phase 1, 3a and 3b and between Phase 1 and Phase 3b for the same age group were performed using a Student's *t*-test for independent samples. In Experiment 2, statistical differences among experimental phases (control, melatonin treatment and post-treatment) were tested using a one-way ANOVA and a Tukey-b post-hoc test for multiple comparisons. In all tests, *P* values below 0.05 were considered to be statistically different. All statistical analyses were performed using SPSS software v. 15.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Rest activity rhythms

N. korthausae of both age groups showed a diurnal RAR in Phase 1 (Table 1, Fig. 1). In general, the adult fish group displayed more robust values for all indices measured, showing higher total activity (657.63 ± 129.94 and 284.44 ± 29.72 counts/day for adults and senescent fish, respectively), with a major percentage of this activity located in the photophase, and more regularity as reflected by the Rayleigh test (r, 0.88 ± 0.04 *vs.* 0.74 ± 0.04). Non-parametric indices showed that the RAR was more stable (IS) in adults than in senescent fish, with a trend toward higher contrast between day and night, expressed as Relative amplitude (RA, 0.83 ± 0.03 *vs.* 0.65 ± 0.07), and lower fragmentation as shown by Intradaily variability (IV, 0.70 ± 0.08 *vs.* 1.01 ± 0.13). Accordingly, the general index of circadian functioning (CFI) was significantly higher for adult fish. About sleep quality, adults had a shorter duration of sleep interruptions. A loss of amplitude in senescent fish RAR can be seen in a mean waveform representation (Fig. 2A).

During Phase 2, a free-running rhythm with a period greater than 24 h from the first days was observed only in adult fish (Fig. 1). There were significant period differences between the two age groups. Adult fish displayed a free-running RAR with a period of 1512.50 ± 6.02 min and a periodogram %V of 23.19 ± 4.01, while the values obtained for these parameters in senescent fish were 1475.66 ± 9.43 min and 13.86 ± 1.21, respectively.

The recovery phase (Phase 3a) showed a rapid entrainment to the initial conditions in the adult fish group, while senescent animals needed more days to entrain their RAR (Fig. 1). Mean waveforms for adult fish reveal a well-defined diurnal pattern, while senescent animals exhibited a more fragmented rhythm with less difference between day and night (Fig. 2B). Data analysis showed that senescent fish had

Table 1
Rest-activity rhythm (RAR) analysis of adult and senescent *Nothobranchius korthausae* subjected to different photoperiod and feeding conditions.

	Phase 1		Phase 3a		Phase 3b		Phase 1 *vs.* phase 3b	
	Adult	Senescent	Adult	Senescent	Adult	Senescent	Adult	Senescent
RAR — general overview								
Total activity (counts/day)	657.63 ± 129.94*	284.44 ± 29.72	338.83 ± 119.30	217.43 ± 105.54	443.85 ± 160.43	170.61 ± 60.22	n.s.	n.s.
Nocturnal activity (%)	12.33 ± 1.48*	19.44 ± 1.91	26.09 ± 4.88	25.74 ± 3.57	21.52 ± 6.78	15.24 ± 2.31	n.s.	n.s.
Mesor (counts/10 min)	3.60 ± 1.29	2.29 ± 0.81	2.35 ± 0.85	1.40 ± 0.68	3.06 ± 1.09	1.19 ± 0.41	n.s.	n.s.
Rayleigh test (r)	0.88 ± 0.04*	0.74 ± 0.04	0.81 ± 0.08	0.75 ± 0.07	0.94 ± 0.03	0.91 ± 0.03	n.s.	+
Circadian/ultradian ratio (%)	84.80 ± 2.25*	64.11 ± 7.22	55.77 ± 11.19	43.69 ± 7.98	78.63 ± 8.23	62.40 ± 6.90	n.s.	n.s.
Periodogram %V	36.35 ± 3.80*	16.73 ± 1.68	31.69 ± 6.33	27.28 ± 2.77	43.36 ± 7.66	31.45 ± 4.66	n.s.	n.s.
Non-parametric indices								
Interdaily stability (IS)	0.42 ± 0.04*	0.18 ± 0.01	0.31 ± 0.05	0.22 ± 0.01	0.43 ± 0.06	0.30 ± 0.03	n.s.	+
Intradaily variability (IV)	0.70 ± 0.08	1.01 ± 0.13	0.91 ± 0.05	1.08 ± 0.09	0.84 ± 0.08	0.99 ± 0.15	n.s.	n.s.
Relative amplitude (RA)	0.83 ± 0.03	0.65 ± 0.07	0.83 ± 0.03*	0.68 ± 0.04	0.77 ± 0.05	0.85 ± 0.04	n.s.	+
Circadian function index (CFI)	0.63 ± 0.03*	0.47 ± 0.03	0.56 ± 0.03*	0.45 ± 0.02	0.59 ± 0.04	0.55 ± 0.03	n.s.	+
Sleep quality								
Sleep interruptions	7.63 ± 1.34	8.5 ± 1.07	8.61 ± 1.16	8.33 ± 1.63	6.71 ± 1.22	6.73 ± 1.62	n.s.	n.s.
Interruption duration (min)	13.48 ± 0.53*	15.43 ± 0.75	14.90 ± 1.64	17.56 ± 3.09	11.94 ± 0.79	12.06 ± 0.81	n.s.	−
Sleep latency (min)	16.33 ± 1.87	13.44 ± 1.58	18.33 ± 5.25	10.31 ± 3.15	13.14 ± 0.95*	9.28 ± 0.80	n.s.	−
Sleep offset (min)	3.55 ± 0.99	7.77 ± 3.18	13.66 ± 6.39	16.00 ± 9.03	5.14 ± 3.12	3.09 ± 1.82	n.s.	n.s.
Rest time (min)	686.85 ± 3.30	684.62 ± 4.38	666.67 ± 14.68	676.12 ± 10.79	689.52 ± 6.27	699.36 ± 4.06	n.s.	+
Sleep efficiency (%)	80.97 ± 2.53	80.60 ± 3.16	74.11 ± 5.11	73.65 ± 6.03	84.05 ± 3.11	84.95 ± 4.09	n.s.	n.s.

All values are presented as mean ± SEM (n = 6). Asterisks denote significant differences between age groups for the same phase when compared using a *t*-test ($P < 0.05$). The right column represents the results of the *t*-test comparison between Phases 1 and 3b for the same age ($P < 0.05$). n.s. = not significant; + = a significant increase in Phase 3b with regard to Phase 1; − = a significant decrease in Phase 3b with regard to Phase 1. r, IS, RA and CFI range values are between 0 and 1. IV is between 0 and 2. Circadian/ultradian ratio = % of variance explained by the first harmonic/Σ % variance explained by harmonics 1 to 6. CFI = (IS + (2-IV)/2 + RA)/3. Sleep efficiency = (Rest time − Sleep interruptions × Interruption duration)/night duration.

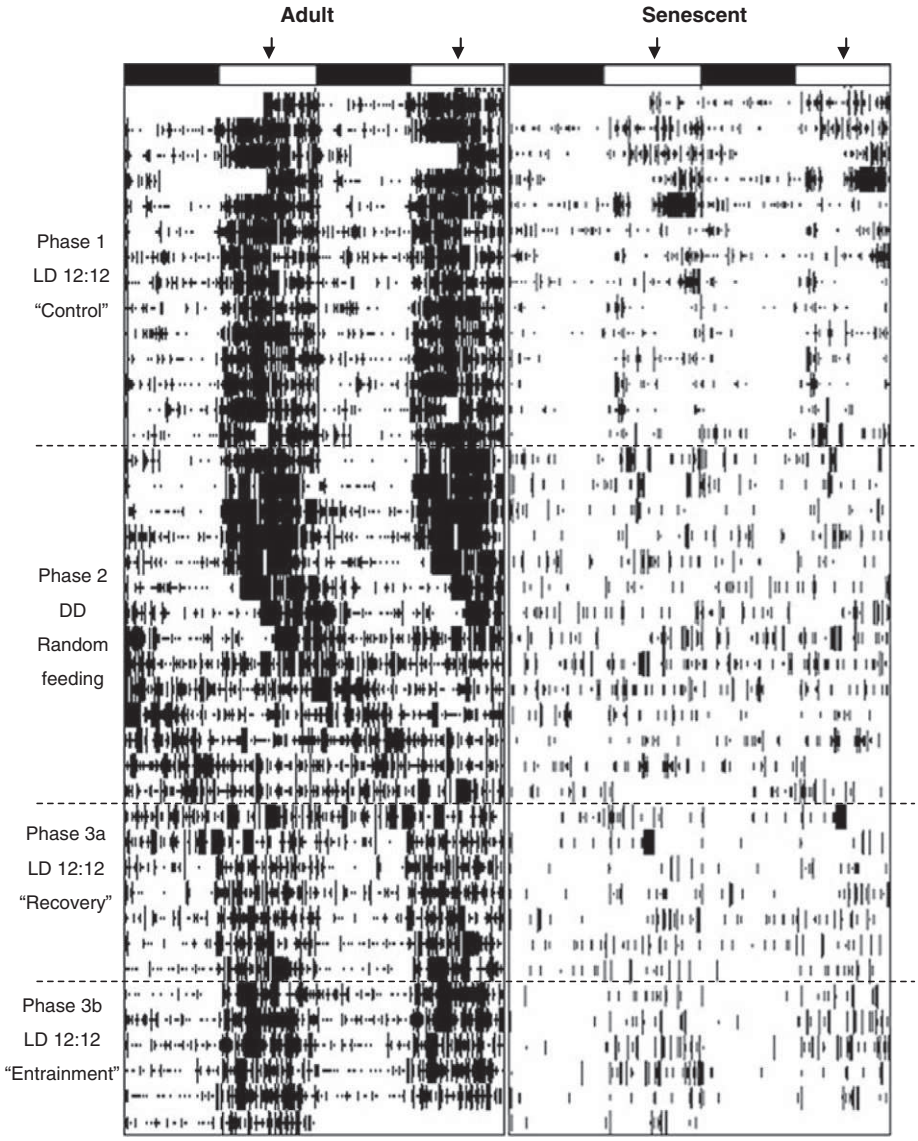

Fig. 1. Two representative actograms showing locomotor activity of adult (left) and senescent (right) *Nothobranchius korthausae*. Experimental periods are indicated on the y-axis: Phase 1 (12L:12D, feeding time at 14:00 h, ZT_6), Phase 2 (DD, random feeding), Phase 3a and 3b (12L:12D, feeding time at 14:00 h, ZT_6). Arrows on top indicate the feeding time for Phases 1, 3a and 3b.

greater difficulties than adults to re-entrain their RAR (Table 1). When both age groups were entrained to the original photoperiod and feeding conditions (Phase 3b), adult fish displayed a RAR similar to that of Phase 1, with no statistical differences. On the other hand, senescent fish showed a RAR improvement with respect to that of Phase 1, with higher regularity (r vector), IS, RA and CFI. With regard to sleep quality in Phase 1 *vs.* Phase 3b, the total duration of the sleep period increased in senescent fish, with a lower average duration of nocturnal sleep interruptions and latency, and no changes in sleep efficiency (Table 1). Finally, as seen in Fig. 2C, an entrainment to feeding time was observed in senescent fish.

3.2. Effects of melatonin treatment on the RAR of senescent fish

Melatonin treatment produced an improvement in senescent fish RAR that can be readily seen in the actograms (Fig. 3). This is also reflected by the increases in robustness (Periodogram %V, 15.95 ± 1.97 *vs.* 27.26 ± 3.74) and regularity (r, 0.76 ± 0.03 *vs.* 0.91 ± 0.01) between Phase 1 and Phase 2, albeit no changes in period and total activity were observed (Table 2). Non-parametric indices and CFI also revealed a significant RAR improvement in response to melatonin in terms of fragmentation (IV, 1.24 ± 0.06 *vs.* 1.03 ± 0.03) and Relative amplitude (RA, 0.65 ± 0.03 *vs.* 0.82 ± 0.02) (Table 2). A significant decrease in nocturnal activity and an increase in feeding time related activity (Fig. 4A and B, Table 2), were also seen with melatonin treatment, as well as an overall improvement in sleep quality with a significant increase in sleep efficiency (from 75.82 ± 1.80 to 82.69 ± 2.27).

After melatonin treatment (post-treatment), several parameters returned to the values of the control phase, including those for nocturnal activity (%), regularity (r vector), non-parametric indices and CFI (Table 2). There was a high feeding time related activity in this Phase although it was lower than in Phase 1 (Fig. 4C).

The acrophase values (maximum locomotor activity time) obtained by a sinusoidal adjustment also changed with melatonin treatment (Fig. 5). A significant reduction in data dispersion during the treatment phase with respect to the control phase (0.50 *vs.* 1.01, $P < 0.05$) can be also seen, indicating that melatonin produced a phase unification. The effect of melatonin persisted for at least 10 days following the end of the treatment, after which a phase delay and a significant acrophase dispersion (1.15, $P < 0.05$) were observed.

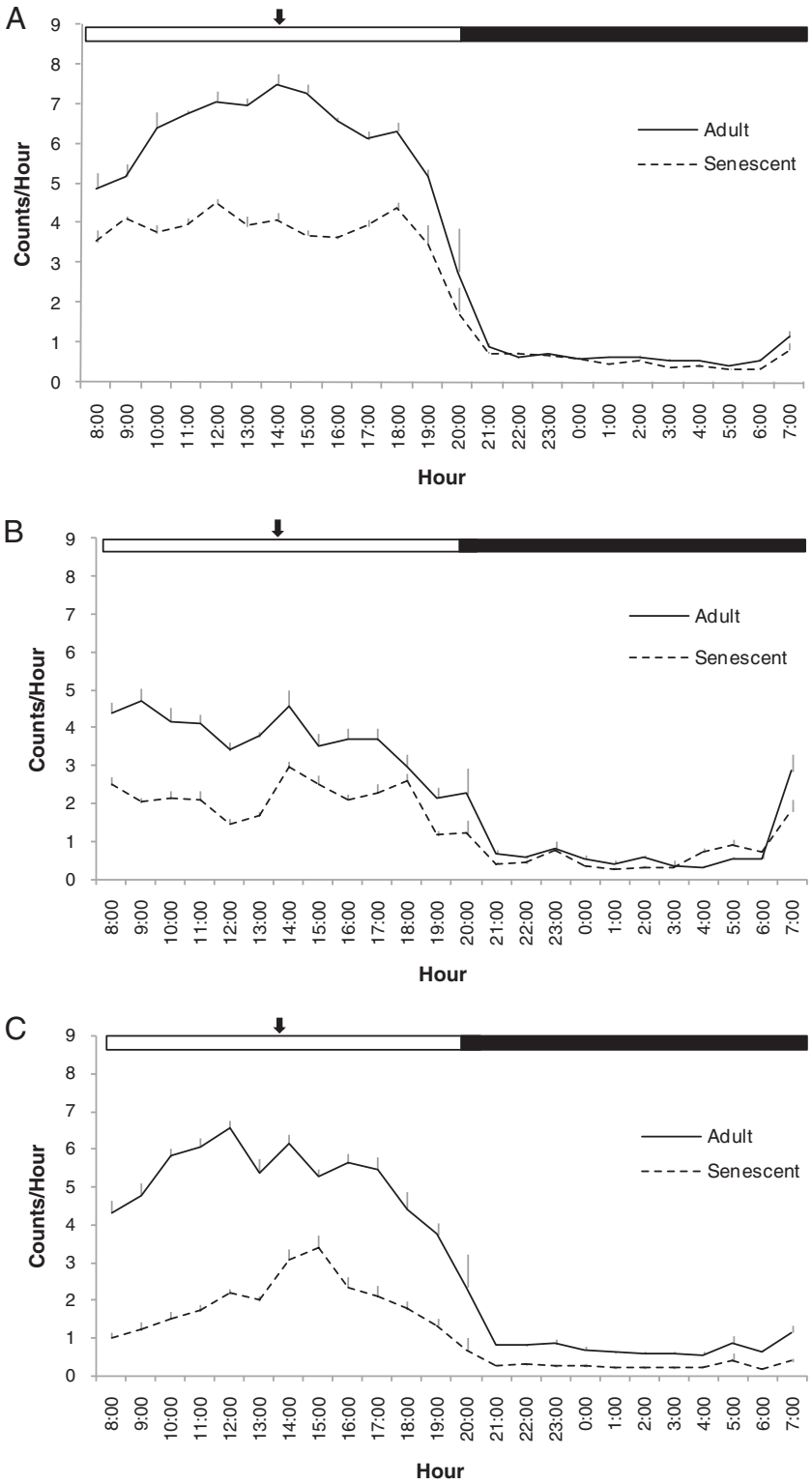

Fig. 2. Mean waveforms obtained for adult (solid line, n = 6) and senescent (broken line, n = 6) fish at different phases of the experiment, presented as mean ± SEM: A) Phase 1, B) Phase 3a and C) Phase 3b. The bar above the mean waveforms represents the photoperiod and the arrow indicates the feeding time.

4. Discussion

The present results indicate that senescence in *N. korthausae* is associated with an impairment of the circadian system (CS). Adult *N. korthausae* showed a more stable rest-activity rhythm (RAR) than senescent animals, in which clear signs of circadian disruption (increased fragmentation, decreased amplitude and regularity, a reduction in total locomotor activity and increased nocturnality) were found. In addition, these changes have been previously characterised as markers of an aged CS in humans and animal models (Weinert, 2000).

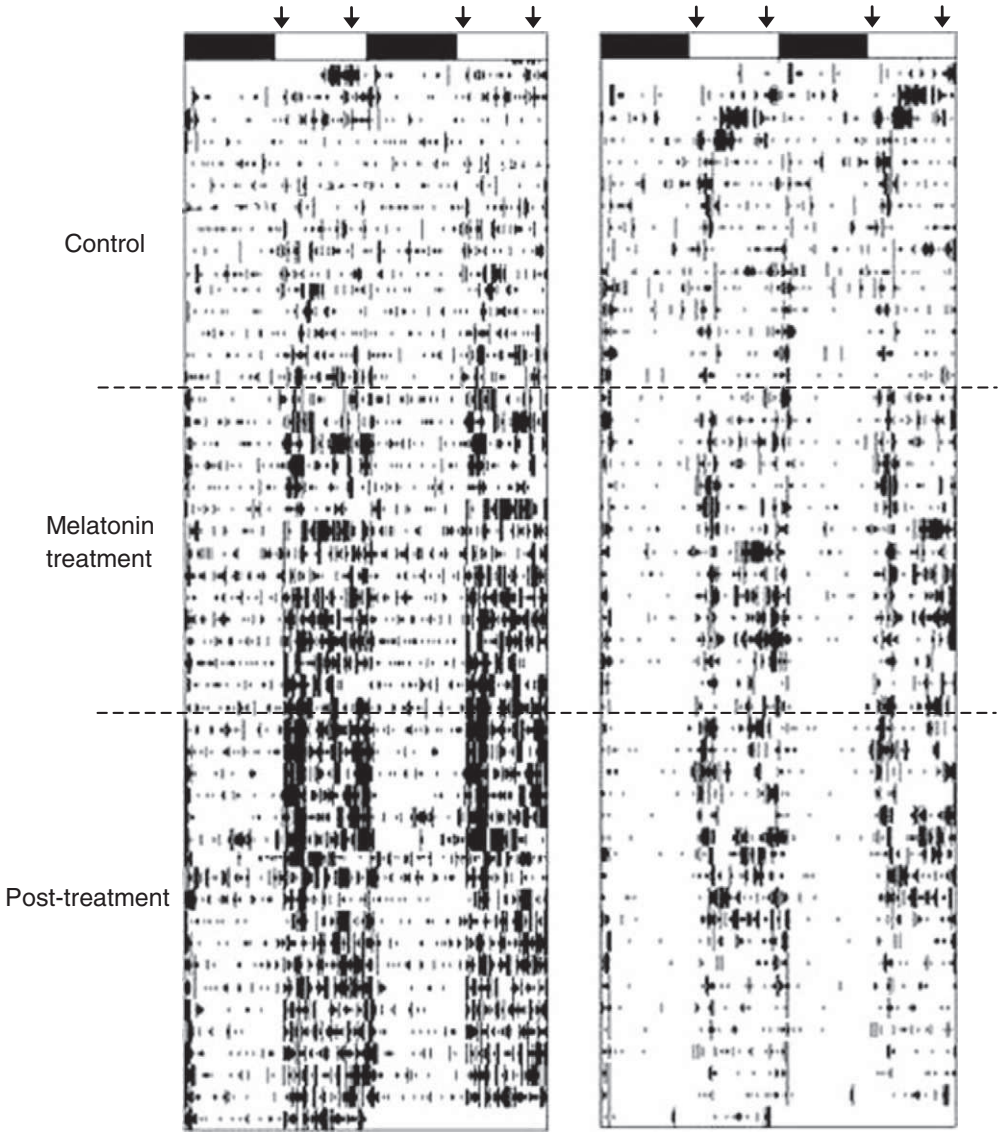

Fig. 3. Two representative actograms showing locomotor activity of senescent *Nothobranchius korthausae* during the melatonin treatment experiment. Photoperiod was set at 12L:12D and feeding time at 10:00 h (ZT_2) and 19:00 (ZT_{11}) (arrows on top). The experiment was divided into 3 phases: control (ZT11 control food), melatonin treatment (ZT11 food supplemented with melatonin) and post-treatment (ZT11 control food).

Moreover, our results also show a shortening of the RAR period (tau) in senescent *N. korthausae* under constant conditions as compared to adult fish. In an environment without external synchronisers (*zeitgebers*), *e.g.* under total darkness and random feeding, circadian rhythms persist with characteristics similar to those entrained, but with a period other than 24 h (see Golombek and Rosenstein, 2010 for a review), which can also be used to determine the status of the circadian system. It has been proposed that a lack of synchronisation between endogenous rhythms and 24-h environmental cycles would imply a physiological cost that would have a detrimental effect on longevity (Wyse et al., 2010). This is supported by a positive link between a tau close to 24 h and the survival rates observed in rodent and primate species (Hurd and Ralph, 1998). However, there is no unanimity in this regard, and some studies have reported no changes in tau with age (Davis and Viswanathan, 1998), a decrease (Kelley, 1988; Pittendrigh and Daan, 1974; Witting et al., 1994) or even an increase (Gutman et al., 2011). Our results show a lower RAR period in the oldest *N. korthausae* under constant conditions, thus indicative of a trend towards a shorter tau with age. The observed changes in the indices (tau value), which define a free-running rhythm, have been considered as markers of central clock alterations in rodents (Pittendrigh and Daan, 1974; Wax and Goodrick, 1975), and to be related to senescence, which would indicate that this is a prevalent aging marker in animals.

Our results show impairment in the capability of senescent *N. korthausae* for entraining to a re-establishment of the light regimen. Senescent fish needed more days to re-entrain to the photoperiod and feeding conditions. When returned to normal conditions after the DD period, senescent fish performed a sequential synchronisation, to feeding first, and to LD cycle later. The feeding schedule seemed to have more relevance as a resynchronising circadian clock than the photoperiod in 72-week-old fish. This fact could be explained by the existence of at least two independent oscillators that are being regulated by the main *zeitgebers*, a light-entrainable (LEO) and a feeding-entrainable oscillator (FEO), as it was showed in the goldfish (Sánchez-Vázquez et al., 1997), which may each be distinctly affected by aging. Age-associated damage affecting the input pathways of photic signals to the circadian system or even the LEO may result in other less-affected oscillators, such as FEO, taking on the responsibility for restoring the rhythmicity of locomotor activity after a period of circadian impairment (Walcott and Tate, 1997). Currently, a large body of evidence links circadian rhythms with metabolism and feeding regimens. These findings prompt the hypothesis that the benefits of the feeding

Table 2
Rest-activity rhythm (RAR) analyses of senescent *N. korthausae* fed control or melatonin-supplemented dried worm diets under the same photoperiod conditions.

	Control	MT treatment	Post-treatment
RAR general overview			
Total activity (counts/day)	242.65 ± 39.59	195.51 ± 34.42	178.28 ± 35.49
Nocturnal activity (%)	25.92 ± 0.81^{b}	21.95 ± 0.76^{a}	28.61 ± 1.23^{b}
Mesor (counts/10 min)	1.38 ± 0.22	1.58 ± 0.33	1.04 ± 0.19
Rayleigh test (r)	0.76 ± 0.03^{a}	0.91 ± 0.01^{b}	0.84 ± 0.04^{ab}
Circadian/ultradian ratio (%)	53.11 ± 4.50	61.77 ± 4.90	51.08 ± 7.42
Periodogram %V	15.95 ± 1.97^{a}	27.26 ± 3.74^{b}	23.51 ± 2.99^{ab}
Non-parametric indices			
Interdaily stability (IS)	0.18 ± 0.02^{a}	0.30 ± 0.04^{b}	0.24 ± 0.02^{ab}
Intradaily variability (IV)	1.24 ± 0.06^{b}	1.03 ± 0.03^{a}	1.28 ± 0.08^{b}
Relative amplitude (RA)	0.65 ± 0.03^{a}	0.82 ± 0.02^{b}	0.71 ± 0.04^{ab}
Circadian function index (CFI)	0.40 ± 0.02^{a}	0.54 ± 0.02^{b}	0.44 ± 0.03^{a}
Sleep quality			
Sleep interruptions	9.09 ± 0.52	7.16 ± 0.82	8.89 ± 1.17
Interruption duration (min)	14.18 ± 0.65	13.36 ± 0.63	14.14 ± 0.86
Sleep latency (min)	14.10 ± 1.81^{b}	5.27 ± 0.97^{a}	13.25 ± 2.29^{b}
Sleep offset (min)	12.90 ± 3.65	10.53 ± 2.63	12.17 ± 4.01
Rest time (min)	675.00 ± 6.92	693.66 ± 5.14	677.63 ± 6.11
Sleep efficiency (%)	75.82 ± 1.80^{a}	82.69 ± 2.27^{b}	75.78 ± 3.44^{a}

All values are presented as mean ± SEM (n = 10). Values on the same line with different superscripts are significantly different (ANOVA, $P < 0.05$) and were obtained by means of a Tukey-b post hoc for multiple comparisons test. r, IS, RA and CFI range values are between 0 and 1. IV is between 0 and 2. Circadian/ultradian ratio = % of variance explained by the first harmonic/Σ % variance explained by harmonics 1 to 6. CFI = (IS + (2-IV)/2 + RA)/3. Sleep efficiency = (Rest time − Sleep interruptions × Interruption duration)/night duration.

regimens could be mediated, at least in part, by a resetting of the circadian clock, thus bringing into synchrony metabolism and physiology (Froy and Miskin, 2010). An increase in acrophase regularity (r vector, concentrated in the feeding time), IS and RA was observed in stabilised senescent fish after the DD period (Phase 3b), as compared to those seen under the initial conditions (Phase 1). The fact that these variables (r vector, IS and RA) may improve after a period of circadian disruption (continuous darkness and random feeding) can be interpreted as a capacity for resilience that allows organisms to cope with stress or adversity.

One of the most important endogenous signals to the organism is the rhythmic secretion of melatonin, which constitutes a chemical signal of darkness. In addition, melatonin has also been described as an antioxidant, immunostimulant, antitumoral and hypnotic molecule (Allegra et al., 2003; Escames et al., 2012; Rodríguez et al., 2004; Sánchez-Barceló et al., 2010). Circadian melatonin production and release are altered with age; the amplitude of the melatonin rhythm decreases and fails to provide enough 'dark' information (Karasek, 2004). This suggests that exogenous melatonin administration may be a potential anti-aging therapy. One possible explanation for the observed weak RARs in senescent *N. korthausae* could be the existence of a deteriorated melatonin rhythm, evidenced by the fact that exogenous administration of this hormone in the diet caused a significant RAR improvement, increasing the rhythm regularity, amplitude and IS and decreasing IV. Moreover, the Circadian Function Index (CFI), as an integrative index, indicated a functional improvement of the circadian system in senescent *N. korthausae*. Exogenous melatonin has been previously reported to be capable of synchronising several endogenous rhythms in both humans and animals (Hack et al., 2003; Lockley et al., 2000; Schuhler et al., 2002; Vivanco et al., 2007). Our results support the hypothesis that melatonin may be useful for restoring circadian systems that are impaired as the result of age.

The sleep enhancing effect of melatonin has also been described in mammals and fish (Zhdanova et al., 2001). Several studies have used fish, mainly zebrafish, as animal models for sleep studies (for review, Zhdanova, 2011), and behavioural criteria have been successfully applied to define sleep in vertebrate species (Campbell and Tobler, 1984). In the present work, we propose to use RAR-derived parameters to develop such an evaluation tool. The parameters that were used to characterise sleep in *N. korthausae* show a slight deterioration during senescence, with a significant increase in the duration of sleep interruptions. It has been established that sleep quality often decreases with age, with this being one of the first processes to be affected in the organism (Myers and Badia, 1995). In addition, some studies have demonstrated that sleep in zebrafish deteriorates with aging, and that this deterioration is partially reverted by the administration of melatonin (Zhdanova et al., 2008). In another study, melatonin administration was shown to be a potential treatment for sleep disorders, such as narcolepsy. Appelbaum et al. (2009) described an interaction between melatonin and the hypocretin system, with melatonin acting as an effective sleep promoter in *hcrt* −/− zebrafish. The present results show that melatonin produces a significant improvement in the sleep parameters of senescent *N. korthausae*, with a decrease in nocturnal activity and an increase in rest time that improves sleep efficiency. This effect disappears a few days after melatonin treatment. Curiously, in our first experiment, only slight differences were observed in sleep quality between 48- and 72-week-old animals. In a previous work, we established that 48-week-old fish are healthy adults and that 72-week-olds are senescent fish at the end of their life-cycle (Lucas-Sánchez et al., 2011). A major deterioration of sleep would be expected closer to death, increasing frailty and compromising several vital processes. It is possible that sleep states in fish suffer a slow, progressive impairment with age, and that a sharp deterioration only occurs when they are close to death.

Considering that aging is a progressive decline of many organic structures and functions, it seems logical to think that the function of the CS may also deteriorate with age. Any worsening of clock inputs and/or outputs of the central clock itself would impair circadian function. One of the main signals from the circadian clock is carried by melatonin acting through MT1 and MT2 receptors, which are widely distributed throughout nervous and peripheral tissues (Falcón et al., 2010). While MT1 activation appears to be more closely related to the amplitude of the rhythms and sleep promotion, MT2 activation has been shown to produce a phase advance (Dubocovich, 2007). In senescent *N. korthausae*, exogenous melatonin caused an increase in amplitude and phase grouping, as well as improved sleep efficiency. When the treatment was stopped, both the amplitude and sleep efficiency returned to their initial values and a phase delay was produced. Our data suggest that the response to melatonin (perhaps due to a normal functioning of their receptors) is not altered in senescent fish, and would seem to point to the inputs and/or melatonin production as the source of the deterioration, since exogenous melatonin re-establishes a normal and stable RAR. This is further supported by reports that aging affects photosensitivity in golden hamsters, reducing the transmission of low wavelength light from the eye lens by 50% (Zhang et al., 1998). A loss in the reception of low wavelength light, the one most closely related to the secretion/inhibition of melatonin (Turner and Mainster, 2008), along with deterioration of the pineal gland (Reiter, 1995) and lens (*e.g.* cataracts) would presumably affect the rhythmic secretion of melatonin compromising the animal's ability to entrain to environmental changes.

In summary, *N. korthausae* suffer a progressive impairment of the CS with age, which can be temporally reverted by melatonin treatment. Aging reduces the amplitude, interdaily stability and tau values of locomotor rhythms, while increasing their fragmentation. All these changes have been previously described as markers of CS dysfunction. Moreover, we have shown that the RAR can be used to characterise sleep-like periods in this species. Sleep deteriorates with age, but this impairment can be reversed by the administration of exogenous melatonin. Therefore, *N. korthausae* is a suitable model to study the aging process by characterising the age-dependent deterioration of the CS. *N. korthausae* suffer from CS age-related changes that can be easily measured and do respond to chronobiotic substances like melatonin.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.exger.2013.02.026.

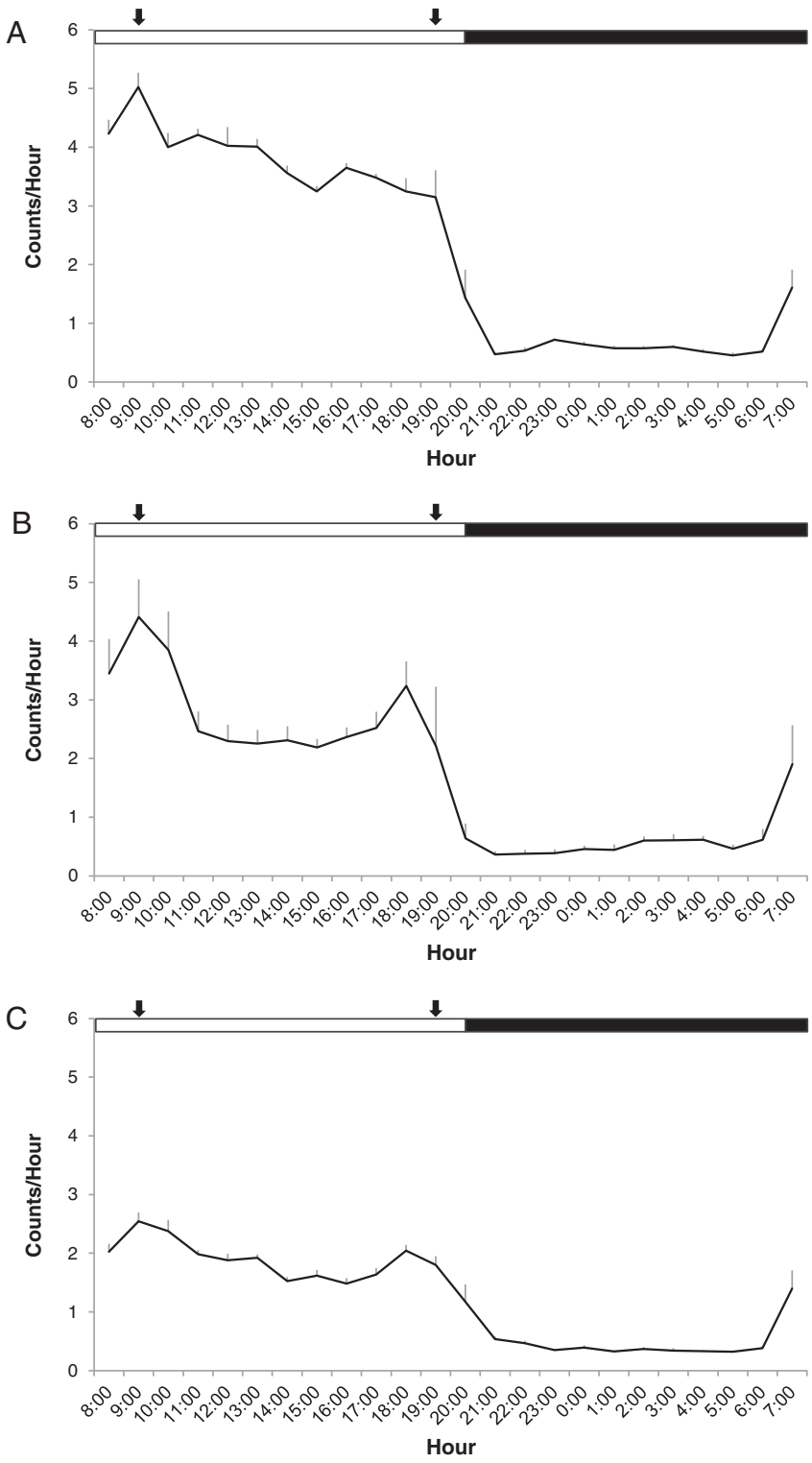

Fig. 4. Mean waveforms obtained from senescent fish during the melatonin experiment presented as mean ± SEM (n = 10): A) Phase 1, control, B) Phase 2, melatonin treatment, and C) Phase 3, post-treatment. The bar above the mean waveforms represents the photoperiod and the arrows indicate the feeding time.

Conflict of interest

The authors declare no conflict of interest.

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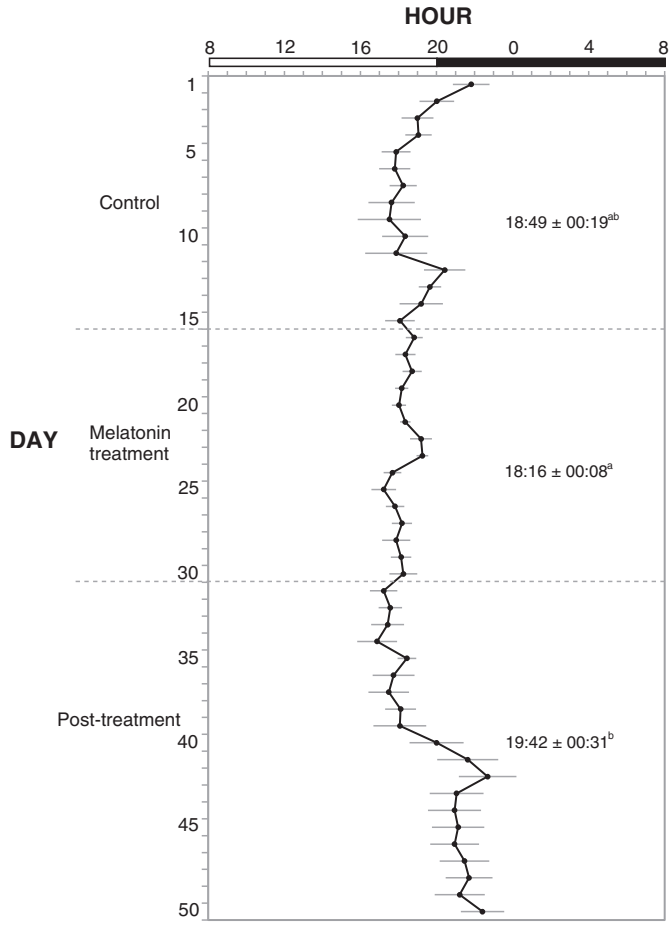

Fig. 5. Daily mean acrophases for locomotor activity of senescent *N. korthausae* during the melatonin treatment experiment: control (15 days), melatonin treatment (15 days) and post-treatment (20 days). Numbers are the mean acrophase in each phase. All data are presented as mean ± SEM (n = 10). Different superscripts indicate statistically significant differences (ANOVA, $P < 0.05$), obtained by means of a Tukey-b post hoc for multiple comparisons test.

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