Circadian timing of inter-bout normothermia during hibernation in European hamsters (*Cricetus cricetus* **L.**)

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Running title Circadian timing of inter-bout normothermia in the European hamster

Abbreviations *CET* central European time, *DD* constant darkness, *DHB* deep hibernation bout, *HB* hibernation bout, *IBN* inter-bout normothermia, *LD* light-dark cycle, *r* mean vector length (correlation coefficient in circular statistics), *LL* constant light, *SHB* short hibernation bout, *SSHB* short and shallow hibernation bout, T_a ambient (cold room) temperature, T_b body temperature, T_m minimal body temperature.

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20 pages, 3 illustrations, and 1 table.

Abstract

Body temperature (T_b) of 3 European hamsters maintained at constant ambient

temperature (T_a = 8 °C) and constant illumination with a dim red light (λ_{max} = 640 nm;

intensity ≤ 1 lux) was recorded throughout the hibernating season using intraperitoneal

temperature-sensitive HF-transmitters. Three types of bibernation bouts (Hbs), i.e. deep

hibernation bouts (DHBs), short hibernation bouts (SHBs), and short and shallow

hibernation bouts (SSHBs), were distinguished due to differences in bout duration and

minimal T_b (T_m).

During prolonged normothermic episodes at the beginning of the hibernation season,

between HBs and at the end of the winter, free-running periods of T_b could be found,

indicating that the circadian system is not arrested during hibernation. In contrast entry

into and arousals from hibernation were widely scattered across day and night and did

not show a clear circadian organization.

Introduction

The hibernation of all animal species investigated so far comprises in hypothermic and normothermic phases (hibernation bouts (HBs) and inter-bout normothermia (IBN)). Due to differences in the duration and the alternating sequence of the two phenomena, a species characteristic pattern of hibernation is generated Lyman (1982), Geiser and Ruf (1995).

The temporal sequence of HBs and IBN in the hibernation patterns appears to be quite coordinated. It is commonly accepted that a complex of endogenous and exogenous factors affects the hibernator and determines the duration of HBs and / or the timing of entries and arousals from HBs, respectively. Most emphasis on external factors was placed on the lighting regime (light-dark cycle (LD), constant light (LL) or constant darkness (DD)) (i.e. Pohl (1967), Daan (1973), French (1977), Canguilhem et al. (1994), Wollnik and Schmidt (1995), Waßmer and Wollnik (1997)) and T_a (i.e. Twente and Twente (1965), Daan (1973), Lindberg (1974), Geiser et al. (1990), Wollnik and Schmidt (1995)).

Almost all of these studies focused on the timing of the HBs; they examined the temporal distribution of the timing of entries into or / and arousals from HBs. Due to the highly variable time course of T_b during both events only discrete points in time during entries into and arousals from HBs were subjected to statistical analyses, although this reduces the available time series essentially. Under a LD-cycle the overwhelming majority of these studies reported a synchronized pattern with certain times of the day of either only entries into HBs or entries into and arousals from HBs. In contrast to these results no daily timing was found under DD or LL.

Just two studies included analyses of continuous recordings of normothermic T_b during IBN under a LD-cycle and received contrary results Wollnik and Schmidt (1995), Waßmer and Wollnik (1997). While the first investigation did not find any rhythmicity in normothermic T_b in between HBs, the second study clearly demonstrated daily rhythms in IBN. In the outdoors enclosure investigation of Wollnik and Schmidt (1995) only very short episodes of IBN were included into the statistical analyses, whereas the interpretation of results in the laboratory study of Waßmer and Wollnik (1997) was complicated by the fact that daily low amplitude oscillations of T_a were present. The first aim of this study was therefore to repeat the analyses of normothermic T_b during IBN under constant conditions in order to search for a circadian rhythmicity. A second aim was to clarify the influence of a LD-cycle on both the timing of HBs and the time course of normothermic T_b during IBN. Because the present investigation was performed under exactly the same housing conditions of European hamsters as in the study of Waßmer and Wollnik (1997) but run under LL, the results of both examinations are ideally comparable.

Materials and methods

Animals and housing

The European hamsters used in this study (two adult males and one adult female) had been obtained from a free ranging population near Strasbourg (France) in late spring. After capture, the animals were kept under standard laboratory conditions (long photoperiod LD 16:8; T_a 20 ± 1 °C) during summer. In October, the hamsters were transferred to a cold room and subjected to constant dim light (LL, $\lambda \approx 680$ nm; intensity < 1 lux until the end of the experiment (April). Implantation of temperature-sensitive transmitters was performed in mid-October about 2-4 weeks before T_a was gradually lowered to 8 ± 1 °C. In the cold room the animals were individually housed in plastic containers of approx. 1m x 1m x 1m size, the top side of which were open and covered by wire mesh. Each enclosure was filled with soil up to 0.2 m as burrowing material and contained various nest boxes made of plastic. Dry hay was offered as nesting material and was replenished once a month. Food pellets (Altromin) and water were given *ad libitum* on a feeding rack and were replenished twice a week at variable times between 08:00 and 16:00 CET. Once in a week additional nutritives such as apples, bananas or nuts were given. Each month the hamsters were weighed and their reproductive status was established under light ether anaesthesia (Ethrane, Abbott GmbH, Wiesbaden, Germany). During the hibernation season this procedure was suspended. Due to the relatively large size of the cage, litter change was not necessary during the hibernation period.

Surgery and temperature telemetry

T_b was measured with temperature-sensitive radio transmitters described in detail by Wollnik & Schmidt (1995). The transmitters (cylindrical shape, diameter 1.5 cm, height 2.5 cm, weight approx. 8 g) were implanted into the peritoneal cavity of each hamster anesthetised by intraperitoneal injections of 50 mg Ketamin (Kemint, Alvetra GmbH, Neumünster, Germany) and 10 mg Rompun (Bayer, Leverkusen, Germany) per kg body weight. For a post-operation period of 10-14 days the animals were housed in polyethylene cages (35 x 55 x 20 cm) at 20 °C and were then returned to the experimental enclosures. No increased morbidity, mortality or other problems resulted from the long-term use of intraperitoneal transmitters.

Statistical analyses

During this investigation, T_b of three hamsters and T_a were continuously monitored with a temporal resolution of 5 min over a period of 4-6 months. All subsequent analyses were based on these 5 min-values. Residual outlying data due to technical or detection failures were rejected by assigning an upper limit to the value of acceptable rates of temperature change. During normothermic days, the upper value was 1°C / 5 min (12°C / h), whereas during hibernating days the upper limit was set between 3-5 °C / 5 min (36-60 °C / h), in order to include the extreme temperature changes during arousals from HBs.

The following parameters were calculated from the temperature recordings during hibernation: (1) time of day at the beginning of each HB (entry), given by the first

measurement of T_b below 30 °C; (2) time of day at the end of each HB (arousal), given by the first measurement above 30 °C.

Normothermia was defined as a body temperature above 30 °C while hypothermia was registered when T_b was below 30 °C.

The presence of circadian rhythmicity during normothermic and hypothermic periods was verified using the chi-square periodogram Sokolove and Bushell (1978).

Pairwise Cross-correlations were conducted according to Box and Jenkins (1976).

Circular statistics (Batschelet 1981) were used to test the significance of timing of entry into and arousal from hibernation. The times of day of these events were converted into degrees, and mean vector directions and lengths as well as circular standard deviations were calculated to describe how well the data coincided. The significance of the computed mean vectors was tested using Rayleigh's test (z-test), while differences between various circular distributions were tested using the Mardia-Watson-Wheeler test.

Results

Types of hibernating behavior and time-pattern of hibernation

In accordance to an earlier study (Waßmer and Wollnik 1997), three types of torpor were distinguished: (1) deep hibernation bouts (DHBs) with T_m below 20 °C and bout duration longer than 24 h; (2) short hibernation bouts (SHBs) with T_m below 20 °C and bout duration shorter than or equal to 24 h; and (3) short and shallow hibernation bouts (SSHBs) with T_m above 20 °C and bout duration shorter than or equal to 24 h. This definition resulted in the classification of 42 DHBs, 5 SHBs and 37 SSHBs. All 3 hamsters showed a regular pattern of DHBs interrupted by episodes of SHBs and SSHBs as well as normothermia (Fig. 1).

Rhythmicity of normothermic body temperature throughout the hibernation period

All three hamsters showed pronounced circadian rhythms in T_b during prolonged normothermic episodes between HBs (Table 1). As an example data are presented for hamster #10 (Fig. 2). From December until March hamster #10 showed only very short episodes of inter bout normothermia besides a longer interruption of the hibernation pattern in the second half of February (Fig. 2A). The periodogram analyses established free-running periods of normothermic T_b in October, November, December and February, ranging from 24.33-25 h (Fig. 2B). Although in December and February periodogram analyses could be calculated by only 2.91 and 4.88 d, respectively, free-running periods of 25 h and 12.25 / 24.58 h could be found. In January and March, when calculation was based on 3.67 and 3.81 d, respectively, no rhythmicity could be

detected. However, pairwise cross-correlation analyses between inter bout

normothermia from different months including January and March revealed significant

cross-correlations ranging from 0.21-0.39 (p < 0.01) indicating a relatively isomorphic

time course of normothermic T_b.

Daily rhythms in the timing of hibernation bouts

According to the Mardia-Watson-Wheeler test, single animals did not show significant

differences in the circular distributions of entries into or arousals from HBs. Therefore

the analyses could be calculated on the pooled data of all three animals. Circular

statistics revealed no significant mean vectors for the timing of entries into nor arousals

from HBs (Fig. 3).

In contrast to previous results under a LD-cycle (Waßmer and Wollnik 1997), no diurnal

or circadian rhythmicity of T_a was present and could obscure the presence of internal T_b

rhythms.

Discussion

Rhythmicity of normothermic body temperature throughout the hibernation period

Detection of free-running rhythmicity in normothermic T_b during inter-bout normothermia (IBN) in almost all months of the hibernation period (Table 1 and Fig. 2) indicates a persistence of circadian timing systems in hibernating animals at least during the normothermic periods.

Only a few other studies focused on the rhythmicity of T_b in-between HBs so far. In a previous laboratory study under a LD-cycle and constant T_a Waßmer and Wollnik (1997) found pronounced 24 h rhythms in the T_b of European hamsters during prolonged normothermic episodes between HBs. These results are highly comparable because IBN was analyzed using the same procedure as in the present study (i.e. chi-square periodogram). Taking into account that all housing conditions but the light regime were identical in both studies, the LD-cycle seems to be the critical variable for the daily pattern of T_b during IBN.

As seen in the study of Waßmer and Wollnik (1997) entrainment of IBN through a LD-cycle was also observed by Pohl (1967) who found that dormice showed a daily periodicity of their midpoint of activity during IBN when kept under a LD. In contrast to both investigations Wollnik and Schmidt (1995) found no evidence of a circadian or dial pattern of normothermic T_b in European hamsters from November until January when kept in an outdoors enclosure. However, due to the very regular hibernation patterns of the outdoors hamsters (i.e. very short IBN), chi-square periodogram analyses of IBN was restricted to very short intervals. Furthermore, combination of various short periods

of IBN in order to create prolonged time-series for the periodogram analyses (Wollnik,

personal communication) may have led a disturbance of the frame-work of the T_b-cycle.

On the other hand, interpretation of the presence of diurnal T_b-rhythms during IBN in

Waßmer and Wollnik (1997) was complicated through the presence of oscillations of T_a

with a τ of 24 h. Although these oscillations were of much smaller amplitude and

showed a different phase as the 24 h rhythms of the inter-bout T_b of the hamsters, there

was still some uncertainty. The exclusion of such regular oscillations under LL and the

occurrence of circadian rhythms in the time-course of T_b during IBN excludes each

possibility of enforced rhythmicity in the present study. It seems very likely that in the

study of Waßmer and Wollnik (1997) the illumination during the LD-cycle provoked the

observed 24 h pattern of T_a, as this rhythm was absent in the present study.

All the other publications which touched the topic of activity or T_b patterns during IBN

(Jansky et al. 1989, Pohl 1987, Fowler and Racey 1990, Grahn et al. 1994) did not use

sufficient sophisticated methods in analyzing IBN to be compared with the presented

results.

Daily rhythms in the timing of hibernation bouts

Under constant illumination and T_a neither the begin nor the end of hibernation bouts

(HBs) showed a daily timing. Again these results could be best compared to the findings

of Waßmer and Wollnik (1997). Kept under exactly the same conditions as in the

presented study but being exposed to a LD-cycle, European hamsters showed a clear

24 h-timing of entries into HBs. This indicates, that the LD-cycle is also involved in the

timing of termination of IBN.

In contrast to the onset of hypothermia, termination of multi-day HBs in the European hamster occurred at any clock hour, even under the presence of a LD-cycle (Canguilhem et al. 1994, Wollnik and Schmidt 1995, Waßmer and Wollnik 1997). The authors of these studies therefore hypothesized that a free-running circadian rhythm which is not effected by the LD-cycle may control the termination of HBs. However, every statistical approach to this problem was unsatisfying so far. The attempt to detect free-running periods underlying the non-24 h pattern of both timing of entries into and arousals from HBs in this study also yielded no satisfactory results. Timing seems to be far from random, but non of the statistical analyses was convincing.

In conclusion, the presented results indicate that the circadian timing system persists during hibernation. Entrainment to 24 h cycles in both IBN as well as the timing of entries into all types of HBs as well as arousals from one-day HBs in the study of Waßmer and Wollnik (1997) can be addressed to the influence of the LD-cycles on hibernating European hamsters.

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Figure captations

Fig. 1

Individual patterns of hibernation in the three hamsters of the investigation. The upper solid line shows the body temperature (T_b) of the animal, the lower dotted line shows the ambient temperature (T_a) of the cold room. The temperature regulation of the cold room failed on November 8th, November 21st and December 25th. The telemetry devices of hamster #9 and #10 failed after March 6th and March 7th, respectively.

Fig. 2A, B

Periodogram analyses of normothermic body temperature during hibernation. (A) The upper solid line shows the body temperature (T_b) of the animal, the lower dotted line shows the ambient temperature (T_a) of the cold room. Monthly intervals used for the periodogram analyses are indicated by circles and ovals. Abbreviations: o (October), n (November), d (December), j (January), f (February), m (March). (B) Monthly chi-square periodograms based on the selected intervals. Next to the abbreviation of the month the number of days used in the calculation of the periodogram is indicated. The solid line shows the amplitudes (Q_p-values) of the periods (τ) between 1-32 h in a steps of 5 min. The dotted line indicates the p = 0,01 significance level of a chi-square distribution based on $12*\tau-1$ degrees of freedom. Peak values of Q_p above the significance level are shown.

Fig. 3A-D

Polar plots of entries into (A, C) and arousals (B, D) from deep hibernation bouts (DHBs) (A,B) compared to short hibernation bouts (SHBs) and short and shallow

hibernation bouts (SSHBs), (C,D). The full circle of 360° represents 24 h in Central European time (CET). Angular data of single animals which did not show significant differences according to the Mardia-Watson-Wheeler test were pooled together. (A) Daily timing of entries into DHBs. (B) Daily timing of arousals from DHBs. (C) Daily timing of entries into SHBs and SSHBs. (D) Daily timing of arousals from SHBs and SSHBs. None of the three hamsters kept under constant illumination and constant T_a showed any significant daily timing of its hypothermic episodes during hibernation.

Table 1: Results of chi-square periodogram analyses on normothermic body temperature

For each hamster and each month of the registration the centred first line shows the number of days used in the calculation of the periodograms. The next line(s) indicate ranges of the period (τ) with Q_p-values above the p = 0.01 significance level. Peak values of τ within these ranges and the corresponding Q_p-value are shown in brackets. Ranges of less than seven 5 min. steps are indicated only by the peak values.

hamster	#8	#9	#10
Oct	10,16d •8,08h (165); 8,5 (190); •23,00-25,92h (24,17h; 742)	10,16d •22,58-26,67h (24,58h; 1217)	10,16d •6,08h (118); 8,17h (267); •11,92-12,33h (12,08h; 365) •16,42h (321) •23,50-25,17h (24,33h; 846)
Nov	7,87d •18,92h (311) •22,00-23,00h (22,67h; 414)	10,19d •22,58-25,42 (23,67h; 922)	5,14d •12,58h (194) •21,17-26,58h (24,42h; 683)
Dec	3,14d •23,75-25,08h (25,08h; 417)	3,09d •16,42-18,00h (16,67h; 326)	2,91d •23,42-25,67h (25,00h; 418)
Jan	1,26d none	3,33d none	3,67d •13,92h (242); 21,00h (322)
Feb	1,74d none	9,06d •13,83h (258) •23,00-24,67h (24,08h; 526)	4,88d •12,25h (206) •23,50-25,42h (24,58h; 480)
Mar	2,42d none	5,50d •22,08-25,67h (24,75h; 488)	3,81d none

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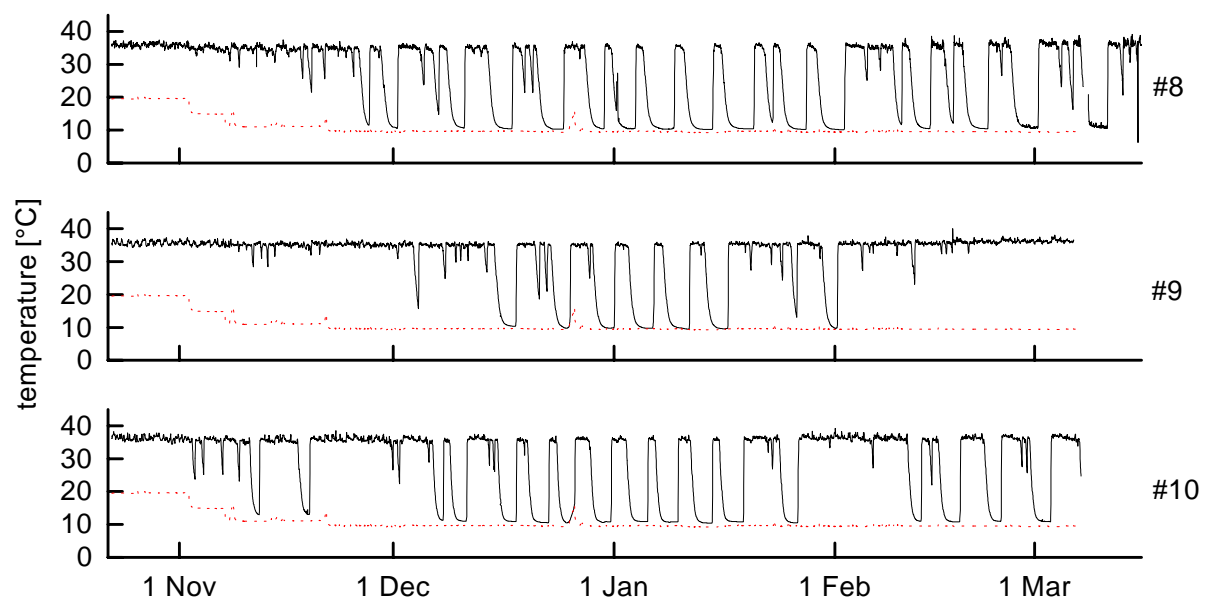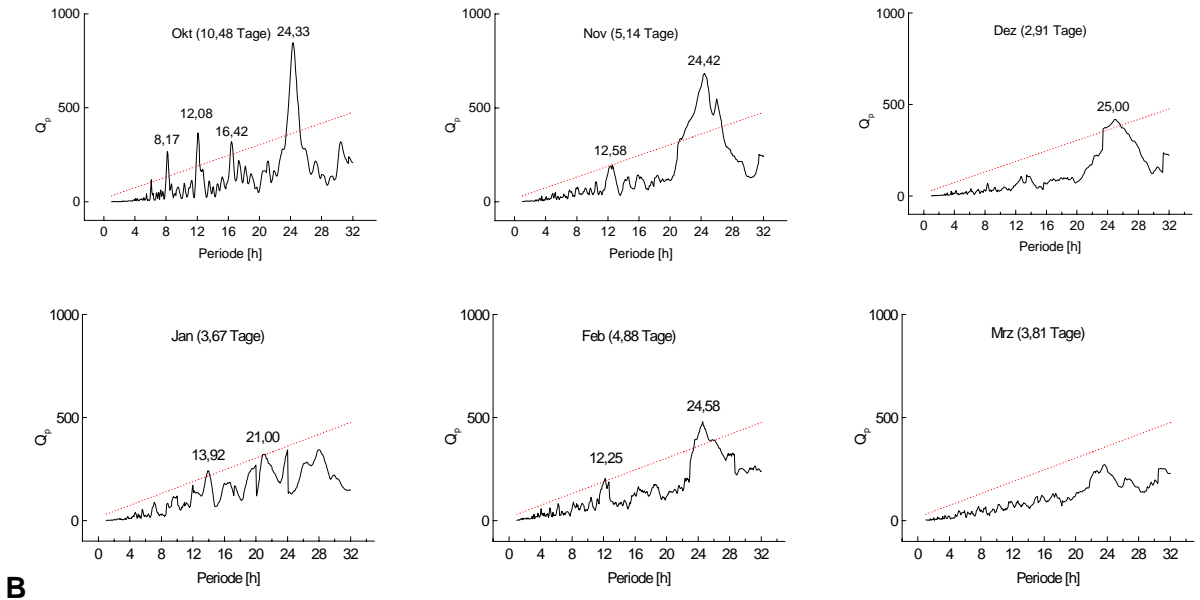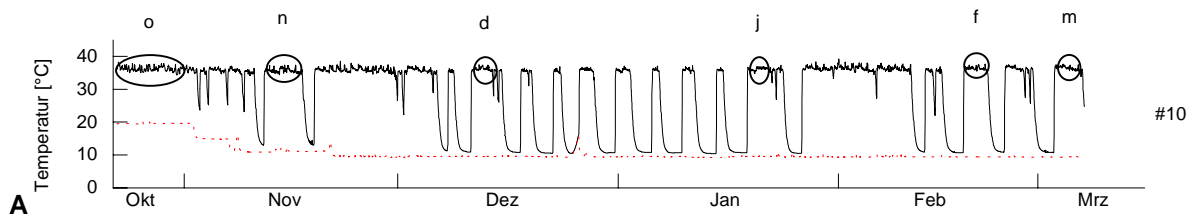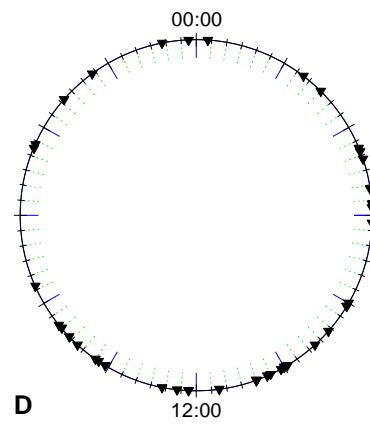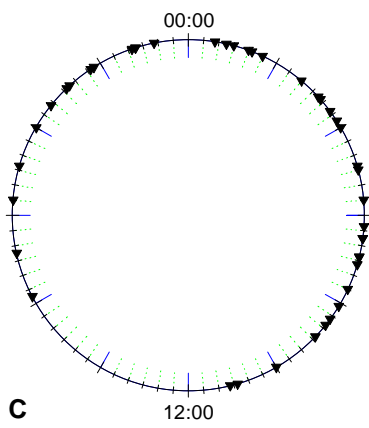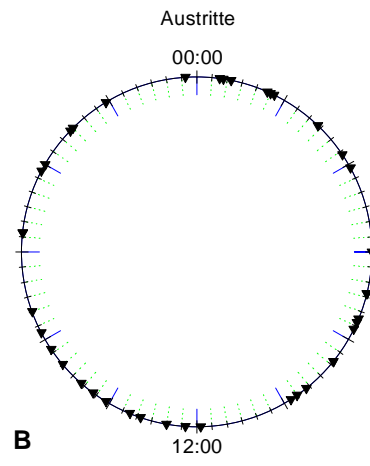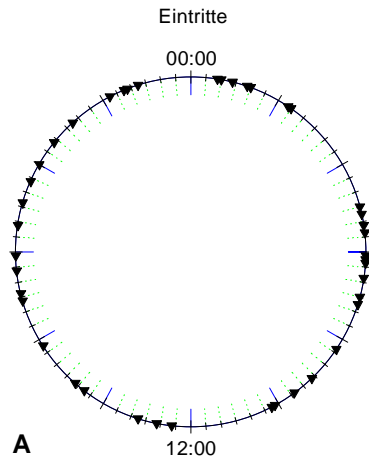
Journal of Biological Rhythms

*Official Publication of the Society for Research
on Biological Rhythms*

June 23, 1998

Dear Dr. Wassmer:

Your manuscript (98-028) entitled "Orcadian timing of inter-bout normothermia during hibernation in European hamsters" has been reviewed for publication in the *Journal of Biological Rhythms* by three referees, and I regret to inform you that it was not accepted for publication. The reviewers raised serious concerns about the manuscript and they did not recommend it highly enough for publication in the journal. The comments of the reviewers are enclosed.

I regret to inform you of this decision.

REVIEWER'S REPORT

EDITOR/ASSOCIATE EDITOR: **REVIEWER#**   **1**

MS#	98-028
AUTHOR(S):	T WaBmer
TITLE:	Circadian timing of inter-bout normothermia during hibernation in European hamsters ...

TO THE REVIEWER:
Use this form for your comments on the manuscript and suggestions for improvement to be transmitted to the author(s). Aoo additional pages if needed and return **(4) copies** to the Editor.

       This paper suffers from several inadequacies that make it unacceptable for this journal. The paper addresses the issue of whether or not circadian body temperature rhythms are present in European hamsters during the normothermic intervals of hibernation. It also addresses whether entry and arousal occurs at regular times of day. To the extent that inferences can be made from so few data, they do support the author's assertion that circadian organization persists during the hibernation season. The second goal of this study is not, however, supported by the data. The author uses time of day to mark the time of entry and arousal from hibernation and then examines those data for the presence of circadian organization. The problem with this is that the animals are free running, as shown in figure 2. Therefore, one cannot conclude that the circadian system does not control timing of entries and arousals based on this analysis. One cannot use time of day as a phase reference point for free-running circadian rhythms. This is exactly the problem that has plagued hibernation research for decades. The only paper I know of that addresses this issue is the Grahn et al. (1994) paper cited by the author. Thus, a similar analysis needs to be performed on these data. Alternatively, the author could try the type of analysis used by Canguilhem et al. (1994), also cited by the author.

       Even if these issues are handled by the author, I'm afraid that several other problems remain. The sample size is unacceptably small, thus, these data need to be included in a future manuscript on this topic. The issue of ambient temperature rhythms confounding such studies is admirably raised here, but no evidence is shown to demonstrate that such rhythms were not a factor in the present study. Less importantly, all figure legends and labels need to be in English for an American journal. This paper also needs major rewriting as well as a more thorough introduction and discussion; these sections present only superficial treatments of the issues relevant to this study. There are also several small problems that need not be enumerated due to the major inadequacies of this manuscript. In sum, the paucity of data, inappropriate data analysis, and superficial treatment of the relevant issues precludes publication of this manuscript.

Comments on the ms. by T Wassmer ("Circadlan timing of inter-bout normotiiermia...")

General:
1.This is the first radiotelemetry study c f body temperature of European hamsters *Cricetus cricetus* hibernating in semi-natural conditions, at a controlled ambient temperature in LL (dim constant red light). Based on 3 individuals of both sexes, it convincingly demonstrates the occurrence of circadian rhythmicity of body temperature in tf ie normomermic episodes between hibernation bouts. Contrary to previous studies performed m other environmental conditions, the author fails to see any circadian dming neither of entry into nor of arousal from hibernation.

2. To me extent thai no physiological specificity of interbour normothermy has eve? be found compared to regular normothermy, the finding of circadian itytamicity is not quite surprising. It had to be experimentally evidenced anyway, and this is the positive aspect of this ms.

3. By contrast, since me animal enters hibernation from normothenny and since this process is thought to share similarities with the induction oi sleep, me absence of rhythmi:ity in toe timing of entries is puzzling and should have beer thoroughly discussed. In view of the difficulties met by the authors of previous studies 10 ascertain the statistical signific :nce of the circadian riming of entries - or of circadian rhythmicity widiin hibernation bouts * it is clear that three animals are much too few to reach any conclusion, especially when using only circular methods which are known to have a low statistical power when used for this purpose. *Any negative conclusion derived from such data is just ill-founded.* It might even be misleading in the future if quoted out of its statistical context.

4. From what is written in *Material and methods* and *Results,* and from Fig. 3, it looks as though only a period of exactly 24 hr has been tested with circular statistics for ctv timing of entries. If this is correct, men how can you expect to tr/jt circadian rhyibmiciry in the timing of entries, once you have previously determined thai the arcadian tau of interbout uormuhermy is generally *different* from 24 hi (see Fig. 2) ?   ,

5. English is poor 10 the point of being hardly understandable at times. For instance, was does the last but one sentence of page 3 mean ("Under a LD cycle... a synchronized pattern with certain times of the day of either entries...") ? The English must be thoroughly checked. preferably by an English or American physiologist.

6. Abstract. "Three types of hibernation bouts were distinguished...". This distinction was not introduced in the present work, but in your paper of 199" with Wollnik. You do not even use it further in the ms. It should therefore be dropped from the abstract.
7. Page 4. Why not quote the beautiful study of Grahn ec al. who first evidenced the circadian rhythmicity of Tb during hibernation bouts ?
8. Page 5. In view of the fast changes in the legal context concerning an endangered species, the year of capture must be indicated.
9. Page 12, ligxie 5. "...every statistical approach 10 this problem was unsatisfying so far". Can you justify this statement ? If not, drop it.
10. Figs. 2 and 3. Labels must be in English.

review of 'Orcadian tuning of inter-bout normothermia during hibernation in European hamsters *(Cricetvs criceiusL.y* by Thomas **Wassmer.**

The author makes the point thai the circadian **system** is **active** within nonnothennic phases during hibernation in hamsters, but not active in tih e timing of the ncrmothennic phases, in **hamsters** in continuous dim red light

There is much clarification needed. The **data** set is **interesting but** minimal three animals were used so the **actual** n=3, although sometimes data were **pooled** for analysis (fig. 3), wkidi is not ideal nor convincing to the reader. A n=3 **calls** for a very **detailed** description and display **of** the data and unambiguous presentation of the **effects.** This is **currently** not the case.

There are many text errors or inconsistencies in th'/ text. For instance, in the abstract fine 2:... with a dim red light... should be... with dim **red** light...

line 3;... throughout the hibernating season ... while **hibernation** was obviously not (spontaneously) ended for 2 out of 3 **animals**

line 4: .. HF-transmitters; it is not clear **what HF means.**

... **hibernation** ... should be **hibernation,** ... Hbs.,, should be... HBs...;

line 6:.. distinguished due to differences... should be... distinguished based on differences...

line **10:** ... the circadian system is not arrested during **hibernation** ... **while** there was **no** circadian . **influence** shown on the timing of normothermic phases and (bus **could** the circadian **system** could have b«en arrested during torpor;

... scattered across day and night... while the experiment **was dc&c** in continuous dim red **light.**

line 12: A wide scatter on a 24h time base does not exclude **circadian** organization. Th& circadian period may be far off 24h **(Grahn** et aL **1994),** and may thus **result** in *a.* wide scatter on **a** 24h **basis.**

These problems occur throughout the maau, cript, and **need** to be corrected.

The presence of circadian rhythmicity in **normothermic** phases is indicated by **periodogram analysis,** in different phases of the hibernation **season.** I d/ nt think that periodogram **analysis** (Sokolove and Bushell 1978) is really **appropriate for** th/, data, since several (most?) data traces were much less than 10 circadian cycles, and probably the Tb signal has a low **amplitude.** Periodogram analysis has trouble detecting periodicity in low amplitude and unstable period signals, It would be better to use an autocorrelation. It also puzzles me why the longer normothermic intervals in figure 2 were not analyzed.

I have problems with the figures. Figure 2 explains what data traces have been **analyzed.** It does however not display any visible indication of circadian **fluctuations.** Figure 1 already shows all the **data,** so figure 2 can be used to indicate detailed inform ation on the circadian **oscillations.** Indicators in some figures are in German, **'temperatur', 'Okt',** 'Dez[1] (figure 3; **'eintritte').**

Figure 3 shows timing of occurrence of different **events** on a 24h real time base for which I see no reason if the author wants to show a circadian based (other then 24h) rhythmic effect. The present analysis of a possible circadian influence on timing of normothennia is in my opinion not correct for free-running conditions, and needs to be **reconsidered.** The only conclusion that can possibly be made with this analysis is that there is no **evidence** hi favor of a 24h rhythmic

component, which in my view is not **that** astonishing, since you **measure** under free-ruaning **conditions.** If it is the **intention** of the author to push the **negative** result, in other words the absence of an effect, I would like to be convinced that it would (in principle) have been possible to detect any effect based on three animals, ihe number of aaimals you base it on is very low.

It would help if the amount of animals should be **increased**. If that is not possible (I appreciate the difficulty of generating this type of data), the **data** should be visualized in such a way that the **effects** found' and 'not found[1] are displayed such that the reader can see from the data what happens and what has been analyzed: periodograms ars OK, but in this case less interesting than actual Tb data showing the circadian Tb patterns. You could show the circadian patterns early and late in hibernation to illustrate the (gradual?) loss of rhythmitity over hibernation.

If It is **the** intention of the author to show a **difference** between LD and DD. this should be addressed more directly. It may be possible to **incorporate** the earlier data, and in **some** way make **statistical** comparisons.

The conclusion that **the circadian timing system persists** during **hibernation (page** 12, line 19) is not valid as such: there may be a Orcadian fluctuation **present** in nonnothennk (this can however not be detected in the late stages of hibernation by the applied periodogram, out needs another trick to be made likely), but there is no effect on the timing c«f nonnothermic phases, A detected gradual loss of circadian rhythnrieaty in the course of **hibernation** in the **absence** of light is in itself interesting, but not considered as a possibility nor **discussed** by the author. The hypothesis that the circadian system is arrested or slowed down at low temperatures should also be discussed.