INFLUENCE OF SEASONAL LIGHT CONDITIONS ON THE PHYSIOLOGY OF ANTARCTIC KRILL, *EUPHAUSIA SUPERBA*

IMPLICATIONS FOR OVER-WINTER BIOLOGY AND MATURITY DEVELOPMENT

DISSERTATION

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"The krill is a creature of delicate and feathery beauty, reddish brown and glassily transparent. It swims with that curiously intent purposefulness peculiar to shrimps, all its feelers alert for a touch, tremulously sensitive, its protruding black eyes set forward like lamps. It moves forward slowly, deliberately, with its feathery limbs working in rhythm and, at a touch of its feelers, shoots backwards with stupefying rapidity to begin its cautious forward progress once again"

Ommunney 1938, cited in Marr 1962 p. 156
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GENERAL INTRODUCTION

Antarctic krill (*Euphausia superba*) is a species which belongs to the order of Euphausiacea within the crustacean superorder Eucarida. Euphausiids, generally referred to as krill, are a group of over 80 species of shrimp-like pelagic crustaceans reaching lengths of 30-150 mm and weights of up to 2 g. The term krill (from the Norwegian word *kril* which refers to small fish) was originally used by North Atlantic whalers to describe the crustaceans found in the stomachs of baleen whales (Nicol 1994). Euphausiid species are widespread in all the oceans of the world with examples occurring from tropical to high polar regions (Siegel 2000; Nicol 2003). Among euphausiids, there are some species that are characterized by high abundance and consequently are of large importance in some marine ecosystems, but also for human exploitation (Everson 2000). Such key species are in particular: *Euphausia pacifica* which is found in the North Pacific Ocean, *Meganyctiphanes norvegica* mainly occurring in the North Atlantic region, and the Antarctic krill *Euphausia superba*, which plays a central role in Southern Ocean waters.

*Euphausia superba* (hereafter krill) is the most abundant of the world’s euphausiids (Fig. 1) and clearly the dominant pelagic crustacean species in the Southern Ocean, with a total biomass of approximately 60-155 million tons (Nicol et al. 2000). Krill grows up to a maximum length of 65 mm (Nicol and Endo 1999) and has a life span of four to seven years (Siegel 2000). Krill ontogenesis from the embryo to the juvenile krill is characterized by a series of larval stages known as nauplius, metanauplius, calyptopis, and furcilia.

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**Fig. 1.** View of Antarctic krill, *Euphausia superba*. (From Stübing, 2004).
Krill holds a dominant position in the Southern Ocean food web, and represents a major trophic link between primary producers and top predators (Fig. 2). On the other hand, krill also plays a significant role in biogeochemical cycles, exporting carbon rapidly to the deep ocean (Bathmann 1992; Nicol 1994). However, although krill is considered to feed mainly on phytoplankton during the productive season, all life stages of krill are known to be capable of switching to alternative food sources, like animal prey or ice biota, when phytoplankton in the water column is getting scarce (Price et al. 1988; Atkinson and Snyder 1997; Perissonotto et al. 2000; Nicol 2006).

![Fig. 2. Simplified food web of the Southern Ocean with the major groups of vertebrate predators centred around krill according to Everson (2000).](image)

Krill has a circumpolar distribution, with main centers of concentration along the continental shelf break and slope, mainly occurring in the upper 200 m of the water column in discrete swarms in densities up to 10,000 to 30,000 ind. m$^{-3}$ (Marr 1962; Quetin and Ross 1991; Nicol 2006). Publications during the last decade have shown that the population size of krill seems to be driven by recruitment, rather than by predation pressure on postlarvae, and that recruitment is suggested to be related to seasonal sea ice dynamics (Smetacek at al. 1990; Siegel and Loeb 1995; Atkinson et al. 2004 Smetacek and Nicol 2005). The relationship is thought to be based on the winter feeding habitat for krill available under sea ice and on the food providing spring bloom after sea-ice melt. High krill concentrations correspond to areas of high summer chlorophyll concentrations and are found particularly in waters east and north of the tip of the Antarctic Peninsula towards the Scotia Sea, the Weddel Sea and South Georgia (Atkinson et al. 2004).
Due to the high biomass of krill (60-155 million tons) compared with the 100 million tons of the world-wide commercial fish resources, it is a new prospective source for the fishing industry. Currently, krill is mainly commercially exploited for the aqua culture and fishing industry and only a small proportion is used for human consumption. However, the biochemical composition of krill (e.g. high protein content, omega 3 phospholipids, astaxanthin, digestive enzymes) has recently received substantial increasing commercial interest (Nicol et al. 2000). The annual landings of approximately 100,000 tons (Siegel 2000) is still relatively low, but in the future, it is most likely that krill will mainly be caught for high quality chemical, pharmaceutical and health products (Nicol et al. 2000). Presently there is very little experience in developing management programmes for a commercial species which constitutes the basis of a food web.

However, in future perspectives, not only the commercial fishery can endanger the krill population and hence the Antarctic marine ecosystem due to its position in the food web. Global climate change appears to cause a decline in the krill population by retreat of winter sea ice (Atkinson et al. 2004). In the productive southwest Atlantic sector which contains > 50% of Southern Ocean krill stocks, the population density has dropped by 80% over the last 30 years with profound implications for the Southern Ocean food web (Reid and Croxall 2001). This part of the Southern Ocean is a fast warming area, and sea ice duration is shortening (Parkinson 2002). Additionally, winter sea ice content is a key factor in high krill densities observed in the southwest Atlantic Ocean. Summer krill densities were shown to correlate to both the duration and the extent of sea ice during the previous winter (Atkinson et al. 2004).

The unique position of Krill in the Southern Ocean food web, the increasing commercial interest on this species, and its susceptibility to global climate change emphasize the urgency of understanding krill life history and biology. Krill is a species with a complex life cycle that has evolved to exploit a highly seasonal environment. One of the crucial questions is how krill is able to adapt to its environment and the forces that determine it.

The main focus of this thesis will be the influence of seasonal light conditions on the physiology of krill, particularly with regard to the over-winter biology and maturity development of krill. The following section will therefore give an introduction to the seasonal development of krill and the major adaptations to its environment. The last part of the introduction will then demonstrate the potential role of the environmental factor light in the life cycle of krill, and will further state the detailed aims of this thesis.
KRILL ADAPTATIONS TO ITS HIGHLY SEASONAL ENVIRONMENT

The Southern Ocean is dominated by extreme seasonal changes in day length, light intensity, sea ice extent and food availability, thus constituting a highly seasonal habitat for krill (Quetin and Ross 1991). The areas south of 60° S, where krill is most abundant, experience a seasonal light regime with a period of near constant light in summer and a period of near constant darkness in winter (Fig. 3). In terms of light incident on the ocean surface, the seasonal cycle of light duration (photoperiod) also defines large seasonal variation in light intensity. Moreover, the area of krill distribution is characterized by the seasonal advance and retreat of sea ice with enormous changes from up to 20·10^6 km^2 at its maximal extent in late winter to 4·10^6 km^2 at its minimal extent in late summer (Knox 1994).

The annual cycles of light and ice coverage shape the annual pattern of phytoplankton concentration in the environment, resulting in an intense seasonality of food supply for krill. The large sea ice cover in winter limits the availability of light to the primary production. Although primary production also exists in winter, phytoplankton concentration in the water column is often < 0.1 µg chlorophyll a (Chl a) L^-1. Sea ice retreat during spring seeds the upper ocean with phytoplankton cells, and creates a low salinity, stable surface layer which leads to a substantial increase of phytoplankton concentration at the ice edge (Constable and Nicol 2003). From December to February, light availability for primary production allows open-water phytoplankton blooms in the range of 10 to 30 µg Chl a L^-1, creating the major food habitat for krill (Knox 1994).

![Fig. 3. Latitudinal variation in day length during the year between 40° and 70° S. (From Knox, 1994).](image-url)
The intense seasonality in environmental features, especially in food availability, implies that the seasonal development of krill also must be seasonal. Krill obviously has evolved a successful and complex life cycle to exploit this highly seasonal environment. The two major characteristics in the seasonal development of krill that guarantee krill’s success are (1) the ability to survive the winter season when much of the Southern Ocean is covered by ice and phytoplankton concentration in the water column is extremely low and (2) the timing of reproduction that allows krill to produce successful offspring in its highly seasonal environment.

Overwintering success of larval and adult krill is a decisive factor that influences krill condition, recruitment, and population size (Siegel and Loeb 1995). However, the physiological mechanisms which enable krill to survive during winter are still poorly known and this topic is characterized by much speculation, few data, and some controversy. Suggested survival mechanisms for adult krill fall into two categories: Firstly, non-feeding strategies and secondly, switching to alternative food sources. Possible non-feeding strategies include the use of stored lipids (Hagen et al. 1996, 2001), reduction in metabolic rates (Kawagushi et al. 1986; Quetin and Ross 1991; Torres et al. 1994), and shrinkage in size (Ikeda and Dixon 1982). Feeding strategies involve switching to ice biota (Marschall 1988), zooplankton (Huntley 1994), or seabed detritus (Kawagushi et al. 1986). All of these overwintering mechanisms have been observed at different times and places, and thus may indicate that krill can utilise a variety of these strategies to cope with the winter situation. However, Quetin and Ross (1991) concluded from their data that a reduction in metabolic rates seems to be the major physiological response to the Antarctic winter, when most of the environment is covered by ice and food in the water is extremely scarce. This issue was also indicated by other studies (Kawaguchi et al. 1986; Torres et al. 1994). Metabolic proxies at molecular (Meyer et al. 2002; Cullen et al. 2003) and overall level (Kawaguchi et al. 1986; Atkinson et al. 2002) have given indications for seasonal variation in the metabolic status in krill. Compared to summer values, several studies also have found low or zero feeding rates during winter, indicating a mixture of reduced opportunistic feeding and reduced metabolism (Morris and Priddle 1984; Quetin and Ross 1991; Torres et al. 1994; Atkinson et al. 2002). Overall, the physiological status of krill varies seasonally with high metabolic and feeding rates in summer merging into low and/or reduced rates in winter, followed by a subsequent increase from winter to summer. This annual rhythm allows krill to allocate metabolic capacity to the times of the year when food is abundant, while switching to an energy-saving mode in winter.
The reproduction of krill is an energetically demanding process that is limited to a short period during the Antarctic summer when environmental conditions are favourable. The onset and timing of maturation is likely to be very important for the reproductive success of krill, and consequently influences the population dynamics of this key species in the Southern Ocean ecosystem (Ross and Quetin 2000). The ovary of female krill begins to mature between September and October, fuelled by the spring bloom available in the lead up to the reproductive season when sea ice melts. The reproductive period is restricted to a 1.5 to 3 month season during the Antarctic summer, alternating with a longer period of gonadal rest (Ross and Quetin 2000). During the reproductive season, female krill undergoes a successive development of ovarian maturation with successive cycles of vitellogenesis, maturation and spawning (Cuzin-Roudy 2000). This cyclic egg production enables krill to recycle the ovary multiple times and produce successive egg batches until the end of the reproductive season. Female krill have been observed to lay up to 3500 eggs in a single batch and may be capable of producing up to nine batches in a season (Ross and Quetin 2000). Gravid krill spawns in offshore waters and the embryos sink to a depth of 700 to 1000 m. After 4 to 6 days the newly hatched larvae (nauplius) swim upward, developing during the so called “developmental ascent” via the metanauplius stage to the calyptopis larvae. The Calyptopis I larvae is the first feeding stage and reaches the surface layer after 30 days. At this point it is critical that the larvae find enough food to continue ontogenesis. The development proceeds via two more calyptopis and six furcilia stages before the larvae metamorphose into juveniles in late winter through early spring. Until the onset of the next reproductive season, krill enter a period of reproductive rest and reorganisation of the ovary. During this period female krill were observed to undergo a progressive development of external sexual characteristics. Following spawning at the end of a reproductive season, the female copulatory organ, the thelycum, regresses from a fully mature stage to an immature stage during the winter period, before once again developing into a fully mature form at the beginning of the next reproductive season (Thomas and Ikeda 1987, Kawaguchi et al. 2006). The maturation of male krill is thought to happen several months earlier in the season and is generally more rapid (Ross and Quetin 2000). Overall, the seasonal cycle of reproduction in krill is synchronized with seasonal cycles of food, ice and light (Quetin and Ross 1991). The development of maturity and the production of eggs, embryos, and larvae are timed so that the first feeding stages of krill develop during the summer phytoplankton bloom rather than during the food-depleted autumn and winter. This enables the larvae to feed immediately and sufficiently after reaching the surface, and thus guarantees the reproductive success of krill.
Altogether, extreme seasonal changes in food availability, sea ice extent, day length, and light intensity dominate the Southern Ocean and shape the seasonal development of krill. In the course of the year, important life-cycle parameters of krill such as metabolic activity and maturity constitute seasonal (annual) patterns that specify the time for reduced metabolic and reproductive activity (Fig. 4). However, up to now the mechanisms causing synchronization between the seasonal development of krill and the seasonal cycles of environmental features are poorly known.

**Fig. 4.** Seasonal cycles of metabolic activity and reproduction in krill synchronized to seasonal cycles of food, ice, and light modified after Quetin and Ross (1991). Graduations in green colour indicate different phytoplankton concentrations in the water column. Bars show oxygen uptake rates per body dry mass (DM) of krill in different seasons in the Lazarev Sea, measured at 0°C (Meyer et al. unpublished data). The oxygen uptake rates in spring/summer (green colour) are significantly different ($p < 0.001$) from the rates measured in autumn (blue colour) and winter (red colour).

**THE POTENTIAL ROLE OF LIGHT IN THE SEASONAL LIFE CYCLE OF KRILL**

Most organisms that inhabit seasonally changing environments have evolved control systems to regulate their physiology by initiating physiological changes due to annual fluctuations of biologically significant factors. Annual biological rhythms are most clearly expressed in organisms inhabiting arctic zones, where seasonal differences in environmental conditions are most pronounced (Gwinner 1986). The critical questions become: which environmental factors are involved in the control of annual rhythms in the life cycle of krill, and how is their influence characterized.
Annual rhythms in life-cycle parameters of krill may be caused simply and directly by the annual cycle of food concentration in the environment, ranging from < 0.1 µg (Chl a) L⁻¹ in winter to > 10 µg Chl a L⁻¹ in summer. Thereby, a reduced metabolism of krill in winter may be generally caused by the scarcity of food at this time of the year and simply reflects a change in ingestion rate. The timing of growth and reproduction would thus reflect energy availability and may be keyed to high food concentrations in the water column, typically occurring during the summer phytoplankton blooms.

However, several studies on live krill demonstrated that annual changes in the physiological status and maturity development of adult krill appeared to be independent of food supply, and more the result of an inherent adaptation rather than an immediate physiological response to ambient food levels. Atkinson et al. (2002) showed that there is a fundamental seasonal transition in krill physiology, irrespective of ambient food levels. Respiration and clearance rates of krill in autumn were only one-third of those during summer, and these did not increase even after 11 days exposure to abundant food. In the same experimental set up during summer, however, krill responded rapidly to high food concentrations (Atkinson and Snyder 1997). In the same context, several experimental studies have observed a successive seasonal krill maturity cycle under constant experimental conditions, indicating that this cyclic event was controlled by an endogenous rhythm apparently not constituted by a changing food supply (Makarov 1975; Denys and McWhinnie 1982; Thomas and Ikeda 1987; Kawaguchi et al. 2006).

Another biologically significant factor in the Southern Ocean environment that varies in an even more regular annual fashion is the natural light regime (see above). The annual course of light intensity and duration can provide reliable and predictive information for the control of physiological processes in krill. In the evolution of control systems of numerous plants and animals, the annual cycle in photoperiod has become the major source of predictive environmental information in the control of a variety of seasonal activities (Gwinner 1986; Brandstätter 2003; Schultz et al. 2003). In general, organisms adapt to seasonal variations in photoperiod by evolving mechanisms to anticipate the succession of the season thereby predicting favourable or unfavourable environmental conditions before they originate. At a simple level of photoperiodic response systems, annual cycles of physiology or behaviour are controlled directly by seasonal changes in the photoperiodic cycle. However, several studies indicate that annual cycles of physiological and behavioural functions often represent endogenous circannual rhythms (Gwinner 1986). Thus, these rhythms appear independent of
direct control of light conditions, and are driven by an endogenous clock which is synchronized with the natural year by an environmental factor, mainly the annual course of photoperiod. Seasonal timekeeping and its associated mechanisms have been investigated in a range of different species and have been reported even in short-lived organisms like insects (Nunes and Saunders 1999). Photoperiod and light intensity have been shown to affect physiological and behavioural parameters in a range of aquatic animals including fish (Biswas et al. 2005), molluscs (Duinker et al. 1999) and turtles (Southwood et al. 2003). To date, however, light as an environmental factor has been given minor consideration as a parameter affecting krill physiological functions.

One possible way of analyzing the influence of light on endogenous functions in krill consists of identifying the substances that are known to play a role in the transduction of light signals. Seasonal changes in the light regime might be transmitted via differences in melatonin secretion in krill, which then influence its physiology. The indoleamine melatonin is suspected to act as a transducer of photoperiodic information (Reiter 1991).

In vertebrates, it is well accepted that the pineal gland, the site of melatonin synthesis, is implicated in conveying photoperiodic information via the daily pattern of melatonin secretion. They have a typical increase in melatonin production during the night, and melatonin is involved in the entrainment of circadian rhythms of behaviour and physiology (Underwood et al. 1987; Reiter 1991). However, it appears that melatonin is an evolutionary conservative molecule with a widespread distribution in the living world. Besides vertebrates, melatonin has been detected throughout phylogeny in numerous non-vertebrate taxa including bacteria, protozoans, macroalgae, vesicular plants, fungi and invertebrates (Vivien-Roels and Pévet 1993, Balzer and Hardeland 1996, Hardeland and Fuhrberg 1996, Hardeland and Poeggeler 2003).

To date, melatonin has also been detected in the visual system of several crustacean species (Hardeland and Poeggeler 2003). Several studies indicate an important role of melatonin in crustacean physiology, and compared to vertebrates, crustaceans show a greater variability in melatonin production. Hereby, crustacean melatonin is not necessarily circadian (Vivien-Roels and Pévet 1986), and if so, not always with a nocturnal maximum (Agapito et al. 1995; Tilden et al. 1997; Tilden et al. 2001). This suggests that a possible seasonal pattern of melatonin secretion in krill could mediate the effect of photoperiodic changes for the organization of annual rhythms in life-cycle parameters of krill. Up to now, the production of melatonin and its influence on physiological mechanisms of krill is unknown.
Krill has evolved a successful and complex life cycle to exploit the highly seasonal environment of the Southern Ocean. In the course of the year, life-cycle parameters of krill such as metabolic activity and maturity constitute seasonal (annual) patterns that guarantee overwintering and reproductive success of krill.

The main focus of this thesis is to investigate if seasonal light conditions in the Southern Ocean affect the physiology of krill and therefore are involved in the control of annual rhythms in the life cycle of krill. Emphasis is put on implications for over-winter biology and maturity development. A further main point is to evaluate the physiological significance of melatonin in krill and its possible role in the transduction of light information.

The aims of this thesis are:

- To examine the effects of different light conditions on metabolic and feeding activity of krill.

- To evaluate the effect of different light conditions on the succession of sexual maturity of krill.

- To prove the occurrence of melatonin in krill.

- To investigate a possible seasonal pattern of melatonin secretion in krill and to derive conclusions about melatonin’s possible role in mediating seasonal metabolic changes of krill.

The results of these investigations are presented and discussed in the form of three manuscripts which are outlined in the next chapter, and finally are summarized at the end of this thesis.
OUTLINE OF THIS THESIS

This thesis investigates the influence of seasonal light conditions in the Southern Ocean environment on the physiology of Antarctic krill *Euphausia superba*. To address this aim the thesis is composed of two complementing levels: (1) laboratory experiments on live krill and (2) analyses of freshly caught krill from two different seasons. In a first step, the effect of different simulated Southern Ocean light regimes on the development of certain life-cycle parameters of krill will be evaluated to examine whether and to what extent the physiology of krill is affected by the seasonal cycle of light conditions. Therefore, experiments were carried out at the Australian Antarctic Division (AAD) research aquarium in Kingston, Tasmania. **Manuscripts I & II** are dealing with this topic. The second step in the thesis will be laboratory analyses of field samples from the Antarctic summer and winter to examine the physiological significance of melatonin in krill. Thus, the metabolic activity were measured in krill from different stations in the Lazarev Sea, sampled during a summer and winter cruise with RV *Polarstern*, and melatonin concentrations in the field samples were investigated to evaluate if seasonal metabolic changes might be mediated via differences in melatonin secretion in krill. The results of these analyses are presented in **manuscript III**.

In **manuscript I**, the effect of different light regimes on feeding activity, oxygen consumption, and activity of the metabolic enzyme malate dehydrogenase (MDH) is studied over several weeks under laboratory conditions. Female and male krill exposed to simulated light regimes of prolonged photoperiod and enhanced light intensity showed an increase in all measured parameters over the experimental period. The results indicate that changes in the environmental light regime have an important effect on physiological parameters of krill such as feeding and metabolic rates and will be discussed in relation to suggested overwintering mechanisms for adult krill.

**Manuscript II** evaluates the effect of different light regimes on the development of sexual maturity and body composition (carbon, nitrogen, lipid and protein) of krill over several weeks under laboratory conditions. It is demonstrated that female and male krill exposed to simulated light regimes of prolonged photoperiod and enhanced light intensity showed an accelerated succession of maturity stages over the experimental period. The possible effect of light on the maturity development and body composition of krill will be considered and the results will be discussed in relation to the effects of simulated light regimes on feeding activity and metabolism of krill available from the previous study.
In manuscript III, the occurrence of melatonin in krill and its possible role in mediating seasonal metabolic changes, is evaluated. The results show seasonal variations in the metabolic status of krill and demonstrate that krill was in a state of reduced metabolic activity during winter. It is further shown that, neither during winter nor during summer, are there detectable melatonin concentrations in krill, indicating that a photoperiodic response system in krill is not based on melatonin as a transducer of photoperiodic information. Possible factors and mechanisms are considered with respect to melatonin determination procedures and photoperiodic time measurement.
LIST OF MANUSCRIPTS

This cumulative thesis is composed of three manuscripts which are already published (manuscript I), under review (manuscript II), or prepared as a draft (manuscript III). Below the manuscripts are listed by numbers and the contribution of all authors is specified.


The concept of this study was developed by M Teschke and B Meyer. Laboratory experiments on life krill were conducted by M Teschke in close cooperation with S Kawaguchi. The manuscript was prepared by M Teschke with contributions by all co-authors.


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The concept of this study was developed by all authors. Laboratory analyses were conducted by C Pape and M Teschke. The manuscript was prepared by M Teschke with contributions by all co-authors.
Simulated light regimes affect feeding and metabolism of
Antarctic krill, *Euphausia superba*

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Abstract
The effect of different light regimes on physiological parameters (feeding activity, oxygen consumption, and activity of the metabolic enzyme malate dehydrogenase [MDH]) of Antarctic krill, *Euphausia superba*, was studied over 12 weeks under laboratory conditions. Krill were exposed to light-cycle regimes of variable intensity to simulate Southern Ocean summer, autumn and winter conditions, respectively using: (1) continuous light (LL; 200 lux max), (2) 12-h light and 12-h darkness (LD 12:12; 50 lux max), and (3) continuous darkness (DD). In all experimental groups, the food concentration was kept at high levels (~ 800 μg C L⁻¹). Krill exposed to LL and LD 12:12 showed an increase in all measured parameters over the experimental period. Physiological parameters of krill held under LD 12:12 showed a more consistent increase and remained below those of krill held under LL. No change was recorded for krill exposed to DD; clearance rates and daily C rations did not respond to the high food availability, and oxygen consumption rates and MDH activity were significantly (*p* < 0.05) lower than those of krill exposed to summer light condition. Thus, changes in the environmental light regime have an important effect on physiological parameters of krill such as feeding and metabolic rates, and may indicate an inherent overwinter adaptation strategy triggered by the Antarctic light regime.
Introduction

Antarctic krill (*Euphausia superba*, hereafter krill) plays a central role in the Southern Ocean ecosystem and is the major trophic link between primary production and vertebrate top predators. Krill have adapted to a habitat with large seasonal changes in food availability, sea ice extent, day length, and light intensity. How krill survive the winter season when much of the Southern Ocean is covered by ice and phytoplankton concentration in the water column is extremely low was one of the key questions studied by the Southern Ocean Global Ocean Ecosystem Dynamics program (SO GLOBEC). Overwintering success of larval and adult krill is a decisive factor that influences krill condition, recruitment, and population size (Siegel and Loeb 1995). Suggested survival mechanisms for adult krill fall into two categories: (1) non-feeding strategies and (2) conversion to alternative food sources. Nonfeeding strategies include the use of stored lipids (Hagen et al. 2001), reduction in metabolic rates (Kawaguchi et al. 1986; Quetin and Ross 1991; Torres et al. 1994), and shrinkage in size (Ikeda and Dixon 1982). Alternative food sources are ice biota (Marschall 1988), zooplankton (Huntley et al. 1994), and seafloor detritus (Kawaguchi et al. 1986).

The reduction in metabolic rates is regarded as one of the most effective energy-saving mechanisms for adult krill during winter (Quetin and Ross 1991). Meyer et al. (2002) found a significant reduction in activity of the metabolic key enzyme citrate synthase (CS) compared to summer values. Likewise, Cullen et al. (2003) found significant seasonal changes in CS activity and RNA:DNA (ribonucleic acid:desoxyribonucleic acid) ratio in krill. The decrease in metabolic rates seems to be the major physiological response of krill to the Antarctic winter, but the mechanisms that cause these reductions are still unknown.

A reduced metabolism of krill in winter may be caused simply and directly by the scarcity of food at this time of the year because food concentration ranges from < 0.1 µg chlorophyll *a* (Chl *a*) L⁻¹ in winter to >10 µg Chl *a* L⁻¹ in summer. However, Atkinson et al. (2002) showed that this may not be the case, as respiration and clearance rates of krill in autumn were only one-third of those during summer, and these did not increase even after 11 days exposure to abundant food. In the same experimental set up during summer, however, krill responded rapidly to high food concentrations (Atkinson and Snyder 1997).

Seasonal differences in the metabolic status may be caused by an inherent annual rhythm which enables krill to adapt to the extreme seasonal changes in food availability between the Antarctic summer and winter and that is keyed to a strong seasonal cue in the Southern Ocean ecosystem. While temperature remains within a narrow annual range (-2 to 2°C), photoperiod and light intensity are environmental parameters with strong seasonality in the Southern
Ocean. The Antarctic light regime in general may have important effects on physiological parameters of krill, such as feeding and metabolic rate. The photoperiod in the high Antarctic ranges from near-constant light in December to near-constant darkness in June, and it could represent a signal for the adjustment of the metabolic status of krill.

The aim of the present work was to study the effect of different light regimes on the metabolism and physiological status of krill. Changes in feeding rates, oxygen consumption, and the activity of the metabolic enzyme malate dehydrogenase (MDH) were recorded weekly during exposure to simulated summer, autumn/spring (hereafter autumn), and winter light conditions in the laboratory. This investigation provides further insights in the survival mechanisms of krill during winter, which are poorly known but are essential in understanding the population dynamic of this key species in the Southern Ocean.

**Materials and methods**

*Sampling and maintaining krill in the laboratory—E. superba* were caught by oblique hauls of a Rectangular Midwater Trawl (RMT 8) in the upper 100 m of the water column in East Antarctica (66° 15′ S, 74° 45′ E, 07 February 2005) during the voyage V3 04/05 with RSV *Aurora Australis*. Immediately after capture, krill were transferred into 200-L tanks located in a temperature constant room at 0°C and dim light (Fig. 1). Each day, 50% of the water was exchanged with fresh pre-chilled seawater to ensure a continual turnover of food and nutrients. Twice a day, dead animals and moults were removed from the tanks.

After arriving in Hobart, Tasmania (17 February 2005), krill were delivered directly to the Australian Antarctic Division (AAD) aquarium and kept in a 1,670-L holding tank (Fig. 1). The holding tank was connected to a 5,000-L chilled sea water recirculation system. The sea water was maintained at 0.5°C and was recirculated every hour through an array of filtration devices. Water quality was monitored continuously. Lighting was provided by fluorescent tubes. A personal computer (PC) controlled-timer system was used to set a natural photoperiod (nLD) corresponding to that for the Southern Ocean (66°S at 30 m depth). Presupposing continuous light and a maximum of 100-lux light intensity at the surface of the tank (assuming 1% light penetration to 30-m depth) during summer midday (December), a sinusoidal annual cycle with monthly variations of photoperiod and daily variation of light intensity was calculated. At the beginning of each month, a new photoperiod was simulated by adjusting the timer system.

One thousand, five hundred krill were fed daily with the following algae at the final concentrations of 1.5×10^4 cells mL⁻¹ of the pennate diatom *Phaeodactylum tricornutum*,
2.2×10^4 cells mL⁻¹ of the flagellate *Isochrysis* sp., and 1.6×10^4 cells mL⁻¹ of the flagellate *Pavlova* sp. (Reed Mariculture). Water flow in the tank was closed (for 8 h) to enable krill to feed on the algal mixture. These algae had been used successfully in several experimental trials at the AAD prior to this study (King et al. 2003), and they guarantee good condition of krill in long-term laboratory experiments with low mortality and high feeding rates.

*Experimental design*—Before starting the light experiments on 30 May 2005, feeding activity in the holding tank was measured (Fig. 1). Additionally, 10 individuals were taken from the holding tank to measure oxygen uptake rates (4 krill) and MDH activity (6 krill). The body length and the digestive gland length were measured in all subsampled krill. These data were used as time zero (t₀) for all experiments.

From the holding tank, 450 krill of mixed sex (mean length ~38 mm) were separated into three cylindrical 100-L tanks (150 krill each) situated within one 1,000-L rectangular container. This system was connected to a 5,000-L chilled sea water recirculation system of the aquarium. The water was maintained at 0.5°C. All tanks within the container had a separate water in- and outflow. The chilled water was simultaneously pumped into the container and into each experimental tank. All tanks drained back to the container, where the water entered into an array of filtration devices. After filtration, the water was pumped back to
the container and tanks. The design of the experimental recirculating facility guaranteed identical water quality and temperature for every experimental stock throughout the study.

Each tank was covered with a black lightproof plastic container with a sliding door at the front side to create a separate light compartment. Lighting was provided by fluorescent tubes (Osram L18W/640 Cool White) covered with a filter film around the outside (ARRI, Marine Blue 131). Photoperiod and light intensity were controlled by a PC-controlled timer system. The three tanks were exposed to one of the following light regimes to simulate Southern Ocean summer, autumn, and winter conditions, respectively: experimental tank (1) summer: continuous light (LL) with a maximum of 200-lux light intensity at the surface of the tank during midday (assuming 20,000 lux at summer sea surface and 1% light at 30-m depth), experimental tank (2) autumn: 12-h light and 12-h darkness (LD 12:12) with a maximum of 50-lux light intensity at the surface of the tank during midday and experimental tank (3) winter: continuous darkness (DD) (Fig. 2).

All three experimental stocks were fed daily with the same algae used in the holding tank at final densities of $3.8 \times 10^4$ cells mL$^{-1}$ for *P. tricornutum*, $9.2 \times 10^4$ cells mL$^{-1}$ for *Isochrysis* sp., and $6.6 \times 10^4$ cells mL$^{-1}$ for *Pavlova* sp. The tanks were checked daily for mortality and moulting. Moults were collected and preserved in 10% formalin solution.

Every week, feeding activity was determined in the experimental tanks by measuring clearance rate and daily carbon ration. Ten krill were subsampled from each tank to measure
oxygen uptake rates (4 krill) and MDH activity (6 krill). Body length and digestive gland length were measured in all subsampled krill. The krill were then stored immediately at -80°C for further analyses.

*Measuring of feeding activity*—Shortly before the algae diet was added into each tank, water flow in the tank was stopped for 5 h to enable krill to feed on the algae mixture. Immediately after adding and mixing the algae diet, three replicate subsamples were siphoned to 250-mL bottles for Chl *a* analysis from each tank to measure the initial concentration of the food. The bottles were incubated in the corresponding tank for the duration that the water flow was stopped in the tanks (5 h). After the incubation time, the water from each tank was gently mixed, and three replicate subsamples were transferred through silicon tubing to 250-mL bottles to measure the final concentration in the tanks. Subsamples of 50 mL from each bottle were filtered onto Whatman GF/F filters, which were incubated overnight in 25 mL of 90% aqueous acetone at 4°C in the dark and centrifuged (700 g) for 3 min. The supernatant was used to measure Chl *a* with a Turner 10-005R fluorometer.

Clearance rates on the total phytoplankton biomass in each tank were calculated by use of the formula:

\[
F = \ln(C_c / C_k) V / m_k t
\]

Where *F* is the clearance rate (mL mg⁻¹ body C h⁻¹), *C_c* is the initial concentration in the tanks, *C_k* is the final concentration in the tanks, *V* is the volume of the tank (mL), *m_k* is the body mass (mg C) of the animals, and *t* is the experimental duration (h).

Ingestion rates were calculated as the product of the clearance rate on the phytoplankton biomass (mL mg⁻¹ body C h⁻¹) and its initial C concentration (mg C mL⁻¹) and then expressed as a daily C ration (% body C d⁻¹) under the assumption that the krill feeding rates reflect the daily average rate. The measured Chl *a* concentrations were converted to C equivalents using a C:Chl *a* ratio of 50 (Meyer et al. 2003).

*Measurements of body length and size of digestive gland*—The body length of subsampled krill was measured from the anterior tip of the rostrum to the posterior end of the uropods, excluding their terminal setae. The digestive gland of subsampled krill was measured through the carapace along their longest axis (Nicol et al. 2004). The length of the digestive gland is expressed as percent of body length.
Respiration measurements—Oxygen uptake measurements were run in filtered seawater (0.1 µm pore size). From each tank, four krill were used and incubated individually in 1.5-L bottles. One bottle of the same volume without krill was used as control for each tank. The krill were rinsed and added to the incubation bottles, which were then topped-up and sealed with parafilm and incubated for 24 h in the experimental tanks. Subsampling at the end of the incubation time was done by inserting a glass tube and siphoning the mixed contents of each bottle into 50-mL Winkler bottles, according to Atkinson et al. (2002). Three replicates were filled for each experimental bottle. Oxygen concentrations were measured after immediate fixation for Winkler titrations as described in Meyer et al. (2002), using a 702 SM Titrino (Metrohm). The decrease in oxygen concentration for all experiments was < 20%.

MDH activity—MDH activity (E.C. 1.1.1.37) was measured in the fifth abdominal segment, which was dissected on ice. The segment was placed into pre-weighted microtubes, and fresh weight (fw) was analyzed by using a microbalance. Extracts of segment tissues were prepared in 500-µL CellLytic™ extraction reagent (Sigma C-2978). Homogenization was performed on ice with a Bio-Vortexer™ for 10 s, followed by sonicating with an ultrasonic cell disrupter using one burst of 10 s. The homogenates were incubated for 15 min at 20°C on a shaker and then centrifuged for 15 min at 14,000 rpm and 4°C. The supernatants were transferred into new reaction cups and stored at -80°C until analysis.

MDH activity was determined as follows: 810 µL reaction buffer (0.1 mol L⁻¹ potassium phosphate, pH 7.0), 30 µL of nicotinamide adenine dinucleotide, reduced form (NADH, 7 mmol L⁻¹ in distilled water) and 30 µL sample were mixed in a semi-microcuvette. After 5 min of pre-incubation at 30°C, the reaction was initiated by adding 30 µL oxaloacetate (12 mmol L⁻¹ in distilled water). The change in absorbance at 340 nm was recorded for another 3 min. The activity was expressed as U g⁻¹fw (µmol min⁻¹ g⁻¹fw) using the extinction coefficient ε₃₄₀ = 6.22 L mmol⁻¹ cm⁻¹.

Statistical analysis—All tests were performed with the computer program SigmaStat 3.00 (SPSS) and the STATISTICA software package 6.1 (StatSoft). While feeding activity in the experimental tanks were measured on the basis of single treatment levels, individual krill were treated as replicates in all other analyses. For analysis of differences in feeding activity among the different light regimes, we explored a relationship between clearance rate and daily carbon ration of krill and time by use of linear regressions. We tested the effect of the different light regimes on feeding activity by analyzing the differences of slopes and
intercepts of the regression lines. Equality of slopes was tested using an F-test, and a Tukey test was performed to detect differences among slopes. The effects of time and light regime on digestive gland length, oxygen demand, and MDH activity were analyzed using a two-way analysis of covariance (ANCOVA) using body length of krill as a covariate, in order to separate the effect of krill size from treatment effect. A Holm-Sidak post-hoc test was used to perform pairwise multiple comparisons to detect temporal significant differences among the light regimes. Data on oxygen demand and MDH activity, initially not normally distributed, were square root transformed and were finally normally distributed. The significance level for all tests was set at $p < 0.05$. Data were expressed as mean ± standard deviation (SD).

Results

**Feeding activity**—The greatest increase in clearance rate was observed in krill exposed to LL followed by krill exposed to LD 12:12 (Fig. 3a, b). Clearance rate ranged from 1.8 to 7.7 mL mg$^{-1}$ body C h$^{-1}$ and 1.8 to 4.6 mL mg$^{-1}$ body C h$^{-1}$ from the beginning ($t_0$-group) until the end of the experiment (12 weeks). Krill exposed to DD showed no increase in clearance rate during the experimental period. Values ranged between 1.1 and 2.5 mL mg$^{-1}$ body C h$^{-1}$ and were only 12% of clearance rate of krill exposed to LL at the end of the study (Fig. 3c).

Krill held under LL showed a strong increase in daily carbon ration, from 1.4% at $t_0$ to 26.3% body C d$^{-1}$ after 12 weeks with a maximum of 41.1% body C d$^{-1}$ in week 11 (Fig. 4a). In krill exposed to LD 12:12, daily carbon ration increased during the first three weeks from 1.4% body C d$^{-1}$ at $t_0$ to 9.1% body C d$^{-1}$, remained constant until week 9, and increased to 16.1% body C d$^{-1}$ at the end of the experiment (Fig. 4b). Daily carbon ration of krill held under DD ranged from 1.4% to 9.8% body C d$^{-1}$, with no distinct changes over the experimental period. At the end of the study, daily carbon ration of krill held under DD was only 12% of that of krill exposed to LL (Fig. 4c).

The analysis of regression lines of temporal changes in clearance rate and daily carbon ration among the different light regimes revealed significant differences of slopes among the different regressions, indicating an effect of light regime.

Krill from the different light regimes showed significant temporal differences in the length of their digestive glands ($p < 0.05$, Fig. 5). The digestive gland length of krill from all three experimental tanks decreased within the first two weeks. However, through the remainder of the experiment a distinct increase in digestive gland length of krill held under LL and LD 12:12 from 9.8±1.3 to 14.5±0.7% body length and from 9.9±0.7 to 13.3±1.0% body length was recorded, respectively. The digestive gland length of krill exposed to DD remained
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**Fig. 3.** Changes in clearance rates of Antarctic krill exposed to a) continuous light (LL), b) 12 hours of light and 12 hours of darkness (LD 12:12), and c) continuous darkness (DD) over an experimental period of 12 weeks. Values represent the mean of 3 replicate subsamples. Slopes among regression are significantly different ($F_{0.05,2,33} = 23.74, p < 0.0001$). LL is significantly different from LD 12:12 ($q_{0.05,33,2} = 4.41$) and DD ($q_{0.05,33,2} = 8.76$). LD 12:12 is significantly different from DD ($q_{0.05,33,2} = 7.09$).

**Fig. 4.** Changes in daily carbon rations of Antarctic krill exposed to a) continuous light (LL), b) 12 hours of light and 12 hours of darkness (LD 12:12), and c) continuous darkness (DD) over an experimental period of 12 weeks. Values represent the mean of 3 replicate subsamples. Slopes among regression are significantly different ($F_{0.05,2,33} = 21.53, p < 0.0001$). LL is significantly different from LD 12:12 ($q_{0.05,33,2} = 5.39$) and DD ($q_{0.05,33,2} = 7.69$). LD 12:12 is significantly different from DD ($q_{0.05,33,2} = 5.38$).
mainly constant, ranging between 8.2±1.2 and 9.6±1.1% body length. After five weeks, and through the end of the experiment, digestive gland length of krill held under continuous darkness was significantly lower ($p < 0.05$) than that of krill exposed to LL and LD 12:12. At the end of the study digestive gland length of krill held under DD was 60% of that of krill exposed to LL.

**Fig. 5.** Changes in digestive gland length (expressed as percentage body length) of Antarctic krill exposed to continuous light (LL), 12 hours of light and 12 hours of darkness (LD 12:12) and continuous darkness (DD) over an experimental period of 12 weeks. Letters indicate significant differences ($p < 0.05$): (a) LL is significantly different from LD 12:12 and DD, LD 12:12 is significantly different from DD; (b) LL is significantly different from DD, DD is significantly different from LD 12:12; (c) LL is significantly different from DD. Vertical bars indicate ± SD ($n = 10$).

**Respiration rates**—Krill exposed to the different light regimes showed significant temporal differences in their mean oxygen uptake rates ($p < 0.05$, Fig. 6). Respiration rates dropped within the first two weeks in all experimental groups. Krill held under LL and LD 12:12 showed a clear increase in their oxygen uptake rates, while respiration rates of krill exposed to DD remained mainly constant, ranging between 22.0±3.1 and 30.0±8.4 µL O$_2$ individual (ind)$^{-1}$ h$^{-1}$. The respiration rates of krill held under LL ranged between 22.9±8.8 and 53.7±12.9 µL O$_2$ ind$^{-1}$ h$^{-1}$ after week 2 until the end of the experiment. In the same experimental period, values of krill held under LD 12:12 were between 26.6±7.3 and 44.7±11.1 µL O$_2$ ind$^{-1}$ h$^{-1}$. After 8 weeks, and until the end of the experiment, respiration rates
of krill exposed to DD were significantly lower \((p < 0.05)\) than the rates of krill exposed to LL and LD 12:12, and were only 61% of that of krill exposed to LL after week 12.

**Fig. 6.** Changes in respiration rate of Antarctic krill exposed to continuous light (LL), 12 hours of light and 12 hours of darkness (LD 12:12) and continuous darkness (DD) over an experimental period of 12 weeks. Letters indicate significant differences \((p < 0.05)\): (a) LL is significantly different from LD 12:12 and DD, LD 12:12 is significantly different from DD; (b) LL is significantly different from DD, DD is significantly different from LD 12:12; (c) LL is significantly different from DD. Vertical bars indicate ± SD \((n = 4)\).

**MDH**—Krill from the different experimental tanks showed no significant temporal differences in their MDH activity until week 10 \((p > 0.05, \text{Fig. 7})\). MDH activity of krill from all experimental tanks ranged between 222.8±32.3 and 291.2±83.5 \(\text{U g}_{\text{fw}}^{-1}\) in the first 6 weeks. From that time on, MDH activity in krill held under LL increased to a maximum of 369.3±78.0 \(\text{U g}_{\text{fw}}^{-1}\) in week 11. In krill held under LD 12:12 MDH activity was highest after 9 weeks \((321.1±93.6 \text{ U g}_{\text{fw}}^{-1})\). In contrast, after 10 weeks, krill exposed to DD showed their lowest MDH activity \((219.3±27.4 \text{ U g}_{\text{fw}}^{-1})\), followed by a slight increase to 244.2±41.4 \(\text{U g}_{\text{fw}}^{-1}\) at the end of the experiment, which was 74% of the MDH activity of krill exposed to LL.

The analysis of covariance of digestive gland length, oxygen demand, and MDH activity using body length of krill as a covariate showed a significant effect and significant interaction of time and light regime for all three dependent variables \((\text{time, light regime, time} \times \text{light regime}, p < 0.001)\), indicating a light regime effect. While there was no significant effect of
body length on digestive gland length of krill at any time within any light regime, body length of krill had a significant effect on respiration rates and MDH activity (body length, $p < 0.001$). However, analysis of covariance of oxygen demand and MDH activity showed no significant interactions between body length and time and light regime, indicating that the effect of light regime did not depend on the level of body length.

**Fig. 7.** Changes in MDH activity of Antarctic krill exposed to continuous light (LL), 12 hours of light and 12 hours of darkness (LD 12:12) and continuous darkness (DD) over an experimental period of 12 weeks. Letters indicate significant differences ($p < 0.05$): (b) LL is significantly different from DD, DD is significantly different from LD 12:12; (c) LL is significantly different from DD. Vertical bars indicate ± SD ($n = 6$).

**Discussion**

Our work has shown the influence of different simulated light regimes on physiological function of *E. superba* for the first time. Although all experimental groups received sufficient food, differences in physiological status related to different light regimes were observed. The results indicate an important effect of the different experimental light regimes, which simulated Antarctic summer, autumn, and winter, and this will be discussed in relation to suggested overwintering mechanisms for adult krill.

The mechanisms for overwintering in krill are still poorly known, and this topic is characterized by much speculation, few data, and some controversy. Suggested survival mechanisms fall into two categories: non-feeding strategies (reduction in metabolic rates,
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starvation, and use of lipid reserves, shrinkage), and conversion to alternative food sources (ice biota, zooplankton, seafloor detritus).

Quetin and Ross (1991) suggested a reduction in metabolic rate as the most important energy-saving mechanism for adult krill in winter. Compared to summer values, several other studies demonstrated reduced metabolic rates and/or low or zero feeding rates of krill at the onset of winter (Atkinson et al. 2002; Meyer et al. 2002) and during winter (Morris and Priddle 1984; Quetin and Ross 1991; Cullen et al. 2003). Torres et al. (1994) suggested a “compromise” overwinter strategy for krill with reduced metabolism and feeding activity that was also found by Atkinson et al. (2002) during an autumn study.

Overall, it remains unclear whether the reduced metabolic rates are caused simply by low food supply and feeding rates in winter, or whether there is a more fundamental seasonal transition in the animal’s physiology, irrespective of ambient food levels. Evidence for the latter was demonstrated by Atkinson et al. (2002). Clearance rates of juvenile and adult krill during an autumn study were < 30% of summer rates and failed to respond even when acclimated to high food concentrations for 11 days.

Our results show that changes of feeding and metabolic activity among the different light regimes are not primarily the result of short-term acclimation to high food concentration. In this study, we exposed krill to simulated Antarctic summer, autumn, and winter light regimes, while the food concentration was kept at high levels (~ 800 µg C L⁻¹) in all experimental groups. Hamner et al. (1983) found that feeding behaviour of krill is affected by several chemical phytoplankton compounds. The authors suggested a chemically induced feeding reaction as an advantageous mechanism to improve food exploitation during the dark period. If this had been the case in our experiments, we would have seen similar responses in all three groups. Clearance rates and daily rations of krill held under DD failed to respond to high food concentrations, and by the end of the experiment, they were only 12% of those of krill exposed to LL. In addition, no increase in size of the digestive gland was recorded. The size of the digestive gland can provide further information on how intensively the krill have been feeding in the previous days and weeks (Nicol et al. 2004).

A similar trend was shown in oxygen consumption rates and MDH activity. These parameters give indications of metabolic changes at overall and molecular level. MDH is a metabolic enzyme that catalyzes reactions in the citric acid cycle and energy metabolism. Activity of MDH shows high positive correlation with oxygen consumption rates and is considered as an effective molecular proxy for metabolic rate (Donnelly et al. 2004). Oxygen
consumption rates were determined to assess the overall metabolic response to simulated light regimes.

Respiration rates and digestive gland length of krill from all three experimental tanks dropped within the first two weeks of the experimental period. Krill might have undergone an initial shock caused by the change of environmental conditions between the holding tank and the experimental tanks, which resulted in a decrease of these two parameters.

We demonstrated that changes in feeding activity and metabolic rates in krill are not simply the result of food supply, but it remains uncertain whether oxygen uptake rates and MDH activity are the result of changes in feeding activity or whether it is the other way around, with feeding rates reflecting changes in metabolic rate. Ikeda and Dixon (1984) suggested that the specific dynamic action (SDA), which is defined as the increase of metabolic activity following ingestion of food, is the major cause of enhanced oxygen uptake of krill. Another explanation could be that increased feeding activity causes enhanced locomotor activity followed by higher oxygen uptake rates and MDH activities. Therefore, the increase in metabolic rates could be interpreted as reflection of enhanced feeding rates.

Controversially, during a field study, Kawaguchi et al. (1986) noted an increase in oxygen consumption of krill correlating with an increase in solar radiation in spite of poor food conditions. This finding would favor the hypothesis that increased feeding activity is caused by enhanced metabolic activity.

Our results indicate that feeding and metabolic activity of krill were affected by the different simulated light regimes. Values of krill held under LD 12:12, and thus exposed to half of daylength and 25% of light intensity compared to LL, showed a more consistent increase, and remained below values of krill held under LL. Krill exposed to DD showed no evident change of parameters. In addition, the digestive gland length of krill exposed to DD at the end of the experiment was still smaller than that of krill at time zero (t0). After the initial decrease of digestive gland length of krill from all three experimental tanks, the gland length of krill exposed to DD should have reached at least a similar size to that of krill at time zero (t0), which was not the case.

The effects of light on feeding activity and metabolic rate of E. superba are unknown. Hirano et al. (2003) demonstrated that maturation and spawning of krill were induced by controlled photoperiod changes during a laboratory study. This indicates that the photoperiod as an environmental factor can influence physiological processes in krill. In general, studies demonstrating the influence of light on feeding rates and metabolism in crustaceans are rare. Fanjul-Moles et al. (1998) demonstrated the effect of light on the neural and endocrine
structures responsible for the control of behaviour and metabolic functions of crayfish during exposure to different photoperiods and light intensities. However, several studies have indicated the relevance of light duration and intensity on diverse other biological processes in crustaceans (Gardner and Maguire 1998; Otero et al. 1998; Hoang et al. 2003). Overall, photoperiod and light intensity have been shown to affect physiological and behavioural parameters in a range of other aquatic animals, including fish (Biswas et al. 2005), molluscs (Duinker et al. 1999), and turtles (Southwood et al. 2003), suggesting the importance of these environmental parameters.

In our study, krill was exposed to different simulated environmental light conditions (summer, autumn, winter). In order to simulate the Antarctic summer and autumn conditions most accurately we adjusted the light regimes to different photoperiods and light intensities (see material and methods, Fig. 2). Consequently, it remains unclear whether the changes in the physiological status of krill were triggered directly by changes of a primary factor, such as light intensity, or triggered by changes of an environmental signal, such as photoperiod. In addition, differences in the physiological status of krill also could have been caused by an internal clock, independent of direct control. In this case, different simulated light regimes may have acted as a synchronizer to adapt the internal clock to the experimental condition. The results from this study, in particular, changes of feeding activity among the different light regimes, showed a relative rapid and straight response to different treatments. This may indicate that changes in the physiological status of krill were triggered directly by the simulated light conditions, rather than by an internal clock mechanism, implicating a more prolonged response. However, to study the response mechanism to differences in light conditions further studies dealing with detailed characterizations on the effects of light are required.

Changes in the light regime might be transmitted via differences in melatonin secretion in krill, which then influence its physiology; the indoleamine melatonin is suspected to act as a transducer of photoperiodic information (Reiter 1991). The presence of melatonin in crustaceans is well documented, and its related enzyme, N-acetyltransferase (NAT), is present within the visual system of several species. Recent studies indicate variable patterns of melatonin production and a variable influence of melatonin in modulating biological circadian and seasonal rhythms in crustaceans (Vivien-Roels and Pévet 1993; Balzer et al. 1997; Tilden et al. 2003). To date, the production of melatonin and its influence on physiological mechanisms and behaviour in Antarctic krill *E. superba* are unknown.
Preliminary melatonin determinations in eyestalks and hemolymph of Antarctic krill have shown concentrations of ~1 pg mg\text{fw}^{-1} and ~0.2 pg µL\text{L}^{-1} immunoreactive melatonin, respectively (unpubl. data). In this study, it remains an open question whether melatonin in krill was involved in initiating physiological changes during the experiment, but the results indicate the presence of a hormone that is involved in conveying photoperiodic information and thus underline the proposed effect of light on the physiology of \textit{E. superba}. Further studies dealing with the nature of this hormone and its mode of action are suggested.

Seasonal changes of the physiological status of \textit{E. superba} have been reported at different times and places. It seems that the decrease in metabolic rates is the major physiological response of krill to the Antarctic winter. The results from this study suggest that seasonal changes in the physiological status of adult krill are more the result of fundamental seasonal adaptations in the animal physiology and behaviour irrespective of ambient food levels. The study underlines the important effect of the Antarctic light cycle on physiological parameters of krill such as feeding and metabolic rates. This may indicate an inherent adaptational overwinter strategy triggered by the Antarctic light regime.

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References


Effects of simulated light regimes on maturity and body composition of Antarctic krill, *Euphausia superba*

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Abstract

The effect of different light regimes on the development of sexual maturity and body composition (carbon, nitrogen, lipid and protein) of Antarctic krill, *Euphausia superba*, was studied over 12 weeks under laboratory conditions. Krill were exposed to light-cycle regimes of variable intensity to simulate Southern Ocean summer, autumn and winter conditions, respectively using: (1) continuous light (LL; 200 lux max), (2) 12-h light and 12-h darkness (LD 12:12; 50 lux max), and (3) continuous darkness (DD). The sexual maturity of female and male krill exposed to LL and LD 12:12 showed an accelerated succession of external maturity stages during the experimental period, while krill exposed to continuous darkness showed no changes in external maturity during the course of the study. Changes in the maturity development of krill between the different light regimes are reflected in changes in body composition. Krill exposed to LL and LD 12:12 showed an increase in lipid utilization, indicating that the development of external maturation may be fuelled preferentially by lipid reserves. In contrast, values of total lipid content of krill held under continuous darkness indicated an unchanged lipid catabolism during the course of the study. Thus, the maturity development of krill was affected either directly or indirectly by the different simulated light conditions. Based on these results, and observations on the effects of simulated light regimes on feeding and metabolic rates of krill available from a previous study, we suggest that the Antarctic light regime is an essential cue governing the seasonal cycle of krill physiology and maturity, and highlight the importance of this environmental factor in the life history of krill.
Introduction

Antarctic krill (*Euphausia superba*, hereafter krill) is a key component of the Southern Ocean pelagic ecosystem. Extreme seasonal changes in food availability, sea ice extent, day length, and light intensity dominate the Southern Ocean and shape the seasonal development of krill. Seasonal cycles of growth and reproduction in krill are synchronized with seasonal cycles of food, ice and light (Quetin and Ross 1991). Krill growth rates correlate with the increase and decline of phytoplankton concentration between spring and late summer (Kawaguchi et al. 2006a). The development of maturity and the production of eggs, embryos and larvae are timed so that the first feeding stages of krill develop during the summer phytoplankton bloom rather than during the food-depleted autumn and winter. Krill also exhibit seasonal changes in the metabolic status. The reduction in metabolic rate over the winter months is regarded as one of the most important energy-saving mechanisms for adult krill during winter (Quetin and Ross 1991; Meyer et al. 2002; Cullen et al. 2003). However, up until now the mechanisms causing synchronization between the seasonal development of krill and the seasonal cycles of environmental features are poorly known.

Generally, krill are thought to show a large potential for physiological plasticity using favourable conditions immediately wherever they are found for growth, reproduction and metabolism (Buchholz and Saborowski 2000). A central question is whether seasonal changes in the life-cycle parameters of krill simply reflect the annual cycle of food concentration in the environment, ranging from $< 0.1 \mu g$ chlorophyll $a$ (Chl $a$) L$^{-1}$ in winter to $> 10 \mu g$ Chl $a$ L$^{-1}$ in summer, or whether there are more fundamental seasonal adaptations in the animal’s physiology, irrespective of ambient food levels, that are driven by other environmental factors.

Changes in the Antarctic light regime were shown to have an important effect on physiological parameters of krill (Teschke et al. 2007). This long-term study in a research aquarium, for the first time demonstrated the influence of different simulated light regimes on the physiology of krill. Although all experimental groups received the same amount of food, differences in feeding and metabolic activity under different light regimes were observed. The study indicates that seasonal changes in the physiological status of adult krill are more the result of fundamental seasonal adaptations in the animal’s physiology and behaviour rather than an immediate physiological response to ambient food levels, which was also indicated by previous studies (Atkinson et al. 2002; Atkinson and Snyder 1997).

However, to date, information on how the environmental light conditions may be linked to the seasonal life cycle of krill is still rare. In particular, the contribution of light conditions on
krill maturity and reproduction is unclear so far, and the data related to this topic are still limited and controversial. Several experimental studies have observed a successive seasonal krill maturity cycle and suggested that this cyclic event was controlled by an endogenous rhythm apparently not governed by light, as this environmental factor remained constant throughout the experimental year (Makarov 1975; Denys and McWhinnie 1982; Thomas and Ikeda 1987, Kawaguchi et al. 2006b). Conversely, Hirano et al. (2003) induced maturation and spawning of krill by controlled photoperiod changes during a laboratory study and suggested that photoperiod plays an important role to adjust the onset of maturity in the wild.

In the highly seasonal Antarctic environment, reproduction of krill is limited to a short period during the Antarctic summer. The onset and timing of maturation is likely to be very important for the reproductive success of krill and consequently influences the population dynamics of this key species in the Southern Ocean ecosystem (Ross and Quetin 2000).

The aim of the present work was to study the possible effect of different light conditions on krill maturity. During a laboratory study (Teschke et al. 2007), differences in the succession of female and male krill maturity stages were observed weekly during exposure to simulated summer, autumn/spring (hereafter autumn), and winter light conditions. Elemental and biochemical composition analyses were carried out on the experimental animals to determine changes in the body composition of krill. This work provides further insights on how the seasonal cycle of krill physiology and maturity are governed by the Antarctic light regime.

Materials and methods
Sampling and maintaining krill in the laboratory

*E. superba* were caught by a Rectangular Midwater Trawl (RMT 8) in the upper 100 m of the water column in East Antarctica (66° 15′ S, 74° 45′ E, 07 February 2005) during the voyage V3 04/05 on the RSV *Aurora Australis*. Immediately after capture, live krill were transferred into 200-L tanks with continuous intact seawater supply, that were located in a temperature constant room at 0°C under dim light (Fig. 1). Once the sea surface temperature increased (beyond 2°C) as leaving Antarctic waters, the continuous seawater supply was cut off and daily exchange of 50% of the water using fresh pre-chilled seawater was conducted. Twice a day, dead animals and moults were removed from the tanks.

After arriving in Hobart, Tasmania (17 February 2005), krill were delivered directly to the research aquarium of the Australian Antarctic Division (AAD) and kept in a 1,670-L holding tank (Fig. 1). The holding tank was connected to a 5,000-L chilled sea water recirculation
system. The sea water was maintained at 0.5°C and was recirculated every hour through an array of filtration devices. Water quality was monitored continuously. Lighting was provided by fluorescent tubes. The light regime was set to a natural photoperiod (nLD) corresponding to that for the Southern Ocean (66°S at 30 m depth) using a personal computer (PC) controlled-timer system. A sinusoidal annual cycle with monthly variations of photoperiod and daily variation of light intensity was calculated by assuming continuous light and a maximum of 100-lux light intensity at the surface of the tank (assuming 1% light penetration to 30-m depth) during summer midday (December). At the beginning of each month, a new photoperiod was simulated by adjusting the timer system.

One thousand, five hundred krill were fed daily with the following algae at the final concentrations of \(1.5 \times 10^4\) cells mL\(^{-1}\) of the pennate diatom *Phaeodactylum tricornutum*, \(2.2 \times 10^4\) cells mL\(^{-1}\) of the flagellate *Isochrysis sp.*, and \(1.6 \times 10^4\) cells mL\(^{-1}\) of the flagellate *Pavlova sp.* (Reed Mariculture). Water flow in the tank was closed (for 8 h) to enable krill to feed on the algal mixture. These algae had been used successfully in several experimental trials at the AAD prior to this study, and they guarantee good condition of krill in long-term laboratory experiments with low mortality and high feeding rates.

![Fig. 1](image_url)  
*Fig. 1.* Schematic representation of experimental krill maintenance from the day of collection (07 Feb 2005) to the end of the experiment (22 Aug 2005). Boxes describe different tanks and the corresponding water volume, water temperature, and light regime. LL; 200 lx max: continuous light with a maximum of 200 lux light intensity at the surface of the tank during midday. LD 12:12; 50 lx max: 12 hours light and 12 hours darkness with a maximum of 50 lux light intensity at the surface of the tank during midday. DD: continuous darkness. nLD: natural photoperiod (see material and methods).
Experimental design

On the 30th May 2005, 450 krill of mixed sex (mean length ~38 mm) were randomly separated from the holding tank into three cylindrical 100-L tanks (150 krill each) situated within one 1,000-L rectangular container (Fig.1). This system was connected to a 5,000-L chilled sea water recirculation system of the aquarium. The water was maintained at 0.5°C. All tanks within the container had separate water in- and outflows. The chilled water was simultaneously pumped into the container and into each experimental tank. All tanks drained back to the container, where the water entered into an array of filtration devices. After filtration, the water was pumped back to the container and tanks. The design of the experimental recirculating facility ensured identical water quality and temperature for every experimental stock throughout the study.

Each tank was covered with a black lightproof plastic container with a sliding door at the front side to create a separate light compartment. Lighting was provided by fluorescent tubes (Osram L18W/640 Cool White) covered with a filter film around the outside (ARRI, Marine Blue 131). Photoperiod and light intensity were controlled by a PC-controlled timer system. The three tanks were exposed to one of the following light regimes to simulate Southern Ocean summer, autumn, and winter conditions, respectively: experimental tank (1) summer: continuous light (LL) with a maximum of 200-lux light intensity at the surface of the tank during midday (assuming 20,000 lux at summer sea surface and 1% light at 30-m depth); experimental tank (2) autumn: 12-h light and 12-h darkness (LD 12:12) with a maximum of 50-lux light intensity at the surface of the tank during midday; and experimental tank (3) winter: continuous darkness (DD) (Fig. 2).

All three experimental groups were fed daily with the same algae used in the holding tank at final densities of $3.8 \times 10^4$ cells mL$^{-1}$ for *P. tricornutum*, $9.2 \times 10^4$ cells mL$^{-1}$ for *Isochrysis sp.*, and $6.6 \times 10^4$ cells mL$^{-1}$ for *Pavlova sp*. The tanks were checked daily for mortality and moulting. Moults were collected and preserved in 10% formalin solution.

Every week, four krill were sub-sampled from each tank to determine the stage of sexual maturity and to measure the body length. The krill were then stored immediately at -80°C for later analysis of elemental and biochemical composition. Before starting the light experiments, four individuals were taken from the holding tank to provide time zero ($t_0$) sexual maturity stage and body composition for all experiments.
Classification of sexual maturity and measurement of body length

Sexual maturity of sub-sampled krill was determined on the morphology of external secondary sexual characteristics. The developmental stages of the thelycum in females and the petasma in males were assessed using a classification system (Table 1) based on Bargmann (1945), and modified by Thomas and Ikeda (1987). This classification provides a more precise description of changes in thelycum development than the system outlined by Makarov and Denys (1981). Morphological examinations were carried out using a stereomicroscope (Leica MZ125).

The body length of sub-sampled krill was measured from the anterior tip of the rostrum to the posterior end of the uropods, excluding their terminal setae (Standard 1, Mauchline (1980)).

Elemental and biochemical analysis

Kril sub-sampled for elemental and biochemical analyses were frozen immediately in liquid nitrogen and maintained at -80°C until analyzed. All composition analyses were conducted on lyophilized individual krill that have been homogenized to a powder. Carbon (C) and nitrogen (N) contents were analyzed in a Euro EA elemental analyzer against an acetanilide standard. Total lipids were extracted with dichloromethane: methanol (2:1, v:v) as described by Hagen (2000), and the lipid content was determined gravimetrically. Protein
contents were analyzed using a standard assay according to Lowry et al. (1951), with bovine
serum albumin as a standard. All contents are expressed in percent of dry weight (% DW).

Statistical analysis

All tests were performed with the computer program SigmaStat 3.00 (SPSS). For all
analyses individual krill were treated as replicates. The effects of time and light regime on
carbon, nitrogen, total lipid and protein contents were analyzed using a two-way analysis of
variance (ANOVA). A Holm-Sidak post-hoc test was used to perform pair wise multiple
comparisons to detect temporal significant differences among the light regimes. The
significance level for all tests was set at $p < 0.05$. Data are expressed as mean ± standard
deviation (SD).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description of 1st pleopod</th>
<th>Description of thelycum</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Undifferentiated 1st pleopod</td>
<td>Thelycum either not visible, or represented only by a straight band across the sternum</td>
</tr>
<tr>
<td>B</td>
<td>Petasma appears as a blunt oval undivided lobe</td>
<td>Two small coxal outgrowths can be distinguished at each end of sternal part</td>
</tr>
<tr>
<td>C</td>
<td>Petasma becomes divided into two lobes, the inner and median lobe</td>
<td>Thelycum half-developed: coxal part larger than sternal part but not heavily chitinized</td>
</tr>
<tr>
<td>D</td>
<td>Wing develops as a fold above the petasma</td>
<td>Thelycum similar to adult shape except smaller and not well chitinized; some pigmentation</td>
</tr>
<tr>
<td>E</td>
<td>Wing grows and curves above the two processes of the inner lobe</td>
<td>Thelycum large and firm (well chitinized); usually coloured bright red</td>
</tr>
<tr>
<td>F</td>
<td>Terminal process of the inner lobe reaches almost the top of the median lobe</td>
<td>Thelycum swollen and red in colour; male spermatophores attached and full</td>
</tr>
<tr>
<td>G</td>
<td>Proximal process of the inner lobe shows a leaf-like expansion at its tip</td>
<td>Thelycum swollen and red in colour; male spermatophores attached but empty</td>
</tr>
</tbody>
</table>

**Results**

Figure 3 shows temporal changes in maturity stages of sub-sampled krill exposed to (a)
LL, (b) LD 12:12, and (c) DD. In total 148 individuals were sampled from the experimental
tanks. The percentage of males was generally lower (28%) when compared to female krill
(72%). Differences in the succession of maturity of female and male krill were observed
between the different experimental tanks.
Fig. 3. Changes in maturity stages of Antarctic krill exposed to (a) continuous light (LL), (b) 12 hours of light and 12 hours of darkness (LD 12:12), and (c) continuous darkness (DD) over an experimental period of 12 weeks. Squares represent maturity stages (see Table 1) with number of individuals inside each square. Dark numbers represent males, white numbers represent females.
While the female krill in all experimental groups were maturity stage D at the beginning of the experiment, those exposed to LL and LD 12:12 advanced to stage E during the experimental period, but only from the week 6 onwards (Fig. 3a,b). In both experimental tanks females with a bright red thelyca (stage E) were first observed in week 6. Thereafter, female krill exposed to LL were in maturity stage D and E for a period of three weeks and then advanced to stage E. After nine weeks, and to the end of the study, all observed female krill exposed to LL remained in maturity stage E (Fig. 3a). On the other hand, changes in female maturity stages held under LD 12:12 were more subtle. After week six and until the end of the study only four krill reached maturity stage E, while the majority of female krill maintained their maturity at stage D (Fig. 3b). In contrast, female krill exposed to continuous darkness showed no changes of maturity over the experimental period (Fig. 3c).

A similar trend in the development of maturity was observed for male krill which were in maturity stage D or E at the beginning of the experiment in all experimental tanks (Fig.3). For krill exposed to LL in total four males with maturity stage F were recorded throughout the course of the study, first observed in week 6 and again in week 11 and 12 (Fig. 3a). Sexual maturity of male krill exposed to LD 12:12 maintained stage E until week 12, when three males that reached maturity stage F were observed for the first time (Fig. 3b). In contrast, no consistent pattern in change of maturity stages was observed for male krill held under DD (Fig. 3c).

The elemental composition (% DW) of experimental krill is shown in Figure 4. Krill exposed to the different light regimes showed significant temporal differences in their mean C content ($p < 0.05$, Fig. 4a). The mean C content at $t_0$ was 51.1±0.8% DW. Once the experiment started a distinct decrease in C content of krill held under LL and LD 12:12 was recorded. Krill exposed to LL showed the strongest decrease to a minimum of 47.2±0.5% DW in week 10, followed by an increase to 48.7±0.7% DW at the end of the experiment. C content of krill held under LD 12:12 decreased to minimum of 48.5±1.3% DW in week 11. In contrast, the C content of krill exposed to DD remained constant through the course of the study, ranging between 50.4±1.8 and 51.5±0.6% DW. After nine weeks, and through the end of the experiment, C content of krill exposed to LL and LD 12:12 was significantly lower ($p < 0.05$) than that of krill exposed to DD.
Fig. 4. Changes in (a) carbon content, (b) nitrogen content, and (c) C:N ratio of Antarctic krill exposed to continuous light (LL), 12 hours of light and 12 hours of darkness (LD 12:12) and continuous darkness (DD) over an experimental period of 12 weeks. Letters indicate significant differences ($p < 0.05$): (a) LL and LD 12:12 is significantly different from DD; (b) LL is significantly different from DD; (c) LL is significantly different from LD 12:12 and DD; (d) LD 12:12 is significantly different from DD. Vertical bars indicate ± SD ($n = 4$).

Fig. 5. Changes in (a) total lipid, and (b) protein content of Antarctic krill exposed to continuous light (LL), 12 hours of light and 12 hours of darkness (LD 12:12) and continuous darkness (DD) over an experimental period of 12 weeks. Letters indicate significant differences ($p < 0.05$): (a) LL and LD 12:12 is significantly different from DD; (b) LL is significantly different from DD; (c) LL is significantly different from LD 12:12 and DD. Vertical bars indicate ± SD ($n = 4$).
No significant temporal differences in the mean N content of krill from the different experimental tanks were observed ($p > 0.05$, Fig. 4b). The mean N content at the beginning of the experiment ($t_0$) was 8.3±0.2% DW. Krill from all experimental tanks exhibited a distinct increase of N content over the course of the study. Krill exposed to LL showed the strongest increase to a maximum of 9.3±0.2% DW in week 11, followed by a decrease to 8.8±0.4% DW at the end of the experiment. A similar trend was shown for krill held under LD 12:12. Values increased to 9.2±0.4% DW in week 12. N content of krill exposed to continuous darkness increased to 8.7±0.2% DW at the end of the study.

The course of C and N content of the different experimental groups is reflected in the course of the C:N ratio (Fig. 4c).

The biochemical composition (% DW) of experimental krill is shown in Figure 5. According to temporal differences in the mean C content, significant temporal differences in mean total lipid contents were found between krill exposed to different light regimes ($p < 0.05$, Fig. 5a). Krill exposed to LL and LD 12:12 showed a distinct decrease of total lipid content from 34.4±1.4% DW at $t_0$ to a minimum of 26.2±1.1% DW (week 10) and 27.3±4.8% DW (week 11), respectively. After then the values from both experimental tanks showed a slight increase until the end of the study. In contrast, total lipid content of krill exposed to DD remained constant through the course of the study, ranging between 32.3±4.8 and 35.7±1.6% DW. After nine weeks, and through the end of the experiment, total lipid content of krill exposed to LL and LD 12:12 was significantly lower ($p < 0.05$) than that of krill exposed to DD (except for LD 12:12, week 10).

There were no significant temporal differences in trends of the mean protein content of krill between the different light regimes ($p > 0.05$, Fig 5b). The mean protein content at $t_0$ was 35.7±0.7% DW. For krill from all experimental tanks a distinct increase of protein content was observed over the course of the study. Krill exposed to LL showed the strongest increase to a maximum of 40.0±1.1% DW in week 11, followed by a decrease to 37.7±1.9% DW at the end of the experiment. A similar trend was shown for krill held under LD 12:12. Values increased to 39.5±2.1% DW in week 12. Protein content of krill exposed to continuous darkness increased to 38.0±0.7% DW at the end of the study.
**Discussion**

In this study, female and male krill exposed to simulated light regimes of prolonged photoperiod and enhanced light intensity showed an accelerated succession of maturity stages over the experimental period. The results of elemental and biochemical body composition analyses revealed enhanced energy requirements of these experimental groups which may have been a reflection of their maturation process. In the following section, we examine the possible effect of light on the maturity development of krill. Moreover, we consider changes in the body composition of krill between the different light conditions. The results from this study will be discussed in relation to the effects of simulated light regimes on feeding activity and metabolism of krill available from the previous study (Teschke et al. 2007).

Reproduction of *E. superba* is limited to a certain period within an annual maturation and regression cycle. The reproductive period is restricted to a 1.5 to 3 month season during the Antarctic summer, alternating with a longer period of gonadal rest (Ross and Quetin 2000). During the reproductive season, female krill undergo a successive development of ovarian maturation, with successive cycles of vitellogenesis, maturation and spawning (Cuzin-Roudy 2000). This cyclic egg production enables krill to recycle the ovary multiple times and produce successive egg batches unless the end of the reproductive season. Until the onset of the next reproductive season krill enter a period of reproductive rest and reorganisation of the ovary. During this period female krill were observed to undergo a progressive development of external sexual characteristics. Following spawning at the end of a reproductive season, the female copulatory organ, the thelycum, regresses from a fully mature stage to an immature stage during the winter period, before once again developing into a fully mature form at the beginning of the next reproductive season (Thomas and Ikeda 1987, Kawaguchi et al. 2006b). The maturation of male krill is thought to happen several months earlier in the season and is generally more rapid (Ross and Quetin 2000).

The results from this study indicate that the maturity development of krill was affected either directly or indirectly by the different simulated light regimes. Sexual maturity of female krill exposed to LL and LD 12:12 advanced from stage D to stage E during the experimental period, while in contrast, female krill exposed to continuous darkness showed no changes of maturity over the experimental period. A similar trend in the maturity development was observed for male krill (Fig. 3).

Thomas and Ikeda (1987) observed a regression of thelycum development over winter with a subsequent redevelopment by the following summer during a one-year laboratory study on
Effects of simulated light regimes...

post-spawned female krill kept in complete darkness, which was consistent with earlier studies (Makarov 1975; Denys and McWhinnie 1982). They suggested that the maturity cycle of krill is controlled by an endogenous rhythm apparently not governed by light, as this environmental factor remained constant throughout the experiment. Similar findings were reported by Kawaguchi et al. (2006b) during a long-term study on the maturity cycle of krill in a research aquarium. The authors highlight the importance of food conditions and suggest that the endogenous cycle of maturity is primarily driven by energy accumulation by krill. The effect of light conditions remains uncertain and the contribution of light is thought to be principally an indirect effect, through regulating the seasonal pattern of primary production. However, Hirano et al. (2003) indicated that maturation and spawning of krill were induced by controlled photoperiod changes during a laboratory study and they suggested that light is an important cue initiating krill maturity.

If the seasonal variation in light conditions is one of the factors controlling or influencing the seasonal maturity cycle as is indicated in our study and in that of Hirano et al. (2003), then this could result from one of (or a combination of) the following: (1) direct response: the onset of krill maturation is governed directly by changes of the absolute intensity of light, and reproduction may start when they are exposed to a certain level of light intensity, (2) signal response: the krill maturity cycle is triggered directly by changes of the light:dark ratio, (3) circannual rhythm: reproduction appears independent of direct control of light conditions and is driven by an endogenous clock which is synchronized with the natural year by an environmental factor, mainly the annual course of the photoperiod.

The observations by Thomas and Ikeda (1987) and Kawaguchi et al. (2006b) clearly indicate that the cyclic maturation process represents an endogenous rhythm, independent of direct control by factors such as light intensity or photoperiod because these environmental factors remained constant throughout the course of the studies. However, this does not automatically imply that light is not necessary for maturation in krill, as was concluded in both studies. If there is a circannual rhythm (type (3) regulation, above) it is possible that the cycle of maturity stages of the experimental krill may have been synchronized with the natural environment by the natural annual light cycle before krill were captured. Given the observations of both studies, the pattern of maturity development in the laboratory in complete darkness thus may represent a free-running circannual rhythm of maturation driven by an endogenous clock.

Assuming that krill maturation undergoes such an endogenous seasonal cyclic event, our study supports the important contribution of light, most likely the course of photoperiod, as an
environmental synchronizing signal, and concurs with the findings of Hirano et al. (2003). Six weeks after krill were segregated and exposed to new simulated light conditions individuals held under summer and autumn light regimes exhibited advanced maturity stages leading to clear differences in krill maturity between the tanks at the end of the study. One explanation of these findings could be that the different photoperiods of the simulated light regimes, once the experimental groups were separated, may have acted as a synchronizer (Zeitgeber) and thus linked the endogenous maturity cycle to the new experimental condition. This may have resulted in an accelerated maturity development under the prolonged photoperiod of the summer light regime simulating the light conditions of the main spawning season. Accordingly, krill held under LD 12:12 showed a more attenuated maturity development, while no changes were recorded for krill exposed to complete darkness.

At the start of our study the experimental krill population already showed relatively advanced maturity stages. Female krill of all experimental groups were in maturity stage D, while male krill were in maturity stage D or E. This indicates that the krill population in the holding tank must have gone through a period of re-maturation before the start of the experiment, synchronized by the prevailing experimental conditions (see Material and Methods).

The specific mechanism of physiological synchronization to light conditions in krill remains unclear at this stage and the physiological components that are involved in the control of an endogenous seasonal maturity cycle has not been identified. Light may affect the maturation process of krill via seasonal patterns of hormone production. Teschke et al. (2007) hypothesized that changes in the light regime might be transmitted via differences in melatonin secretion in krill which then influences their physiology. The hormone melatonin, known as a transducer of the photoperiodic message in vertebrates, has also been detected in the visual system of several crustacean species (Hardeland and Poeggeler 2003). Although, most of the studies showed that melatonin is secreted with a marked circadian rhythm (e.g. Balzer et al. 1997; Tilden et al. 2001), melatonin production has also been shown to exhibit important seasonal changes (Vivien-Roels and Pévet 1993). This suggests that a seasonally changing profile of melatonin secretion could also convey photoperiodic information for the organization of a circannual rhythm. The production of melatonin and its influence on physiological mechanisms and behaviour in Antarctic krill has not been studied but our results indicate that there may be utility in further studies dealing with the nature of this hormone and its mode of action in krill.
Light may also have an indirect effect on the maturation cycle through regulating other seasonal physiological processes in krill, which then affect the maturation development (Teschke et al. 2007). During the course of this earlier study, the simulated summer and autumn light regimes caused a successive increase of clearance rate and daily carbon ration of krill, while there was no evident of such changes in krill exposed to winter light conditions. This increasing energy accumulation may then have accelerated the succession of krill maturity kept in these tanks, while krill exposed to continuous darkness accumulated less energy thus the maturation process did not proceed as fast as in the other groups. This agrees with the findings of Kawaguchi et al. (2006b) showing that well fed krill exhibited earlier recovery of maturity stages compared to starved animals. Food availability plays an important role for krill reproduction; food must be available at the right time and in sufficient amounts to satisfy the energetic demands of reproduction (Ross and Quetin 2000). The results from the current, and the earlier study (Teschke et al., 2007), suggest that the Antarctic light cycle may also affect the maturity development of krill indirectly through regulating seasonal patterns of feeding activity and energy accumulation.

At no point during the experimental period was any ovarian growth or development observed in female krill that had showed an accelerated succession of external maturity. In addition, no mating or spawning events were observed in the tanks. Beside the fact that our observation period was too short to demonstrate such development there is a possibility that the ovarian maturity of krill needs additional resources or environmental cues for their further development and these may have been missing in our experimental approach.

Although the sample size in our study was small and the observation period was limited, the results suggest that light as an environmental factor either directly or indirectly affected the maturation process of krill. However, further studies dealing with detailed characterizations on the effects of light are required to assess how essential this environmental influence is, and to understand which specific process may be regulated by light conditions.

Changes in the maturity development of krill between the different light regimes were reflected by differences in the biochemical composition, demonstrating an increase in lipid utilization of krill exposed to LL and LD 12:12 until the end of the experiment (Fig. 5). These trends were confirmed by changes in the elemental composition and the corresponding C:N ratio (Fig. 4). Elemental analyses are often used for indirect estimates of the proximate biochemical composition, and the C:N mass quotient reflects changes in the relative proportions of lipids and proteins (Anger and Harms 1990).
Both the biochemical and elemental composition measured in this study may reflect different energy requirements of krill held under the simulated summer, autumn and winter light regimes. It is likely that the accelerated maturity succession of krill exposed to LL and LD 12:12 have caused enhanced energy expenditures and that, in spite of an increasing daily carbon ration, body lipids were used to fulfill these energy requirements. Temporal changes in biochemical composition, body length and energy accumulation of krill over the experimental period suggest that krill exposed to LL and LD 12:12 used lipid reserves to balance the energy budget (Fig. 6). Although, the phytoplankton concentration during the course of the study was high (~ 800 µg C L⁻¹) it is possible that the food conditions did not represent a sufficient level to satisfy the large energy requirements of the maturation process. The composition of phytoplankton was limited compared to the natural environment and no additional energy rich food (e.g. copepods) was added. The energy intake of krill, at least during the start of the experiment, might not have been high enough to balance the cost of maturation in addition to increasing expenditures for feeding activity and metabolism (see Teschke et al. 2007). This may have caused a shortage of energy accumulation and resulted in the utilization of body lipids. Another possible explanation may be that krill at the start of the study represented a high level of lipid accumulation, which enabled them to preferentially fuel the energy-demanding acceleration of maturity by utilizing these large energy reserves, whereas the energy gain from assimilated food was used to preferentially accumulate body protein (growth). Percentage changes in the total protein content and body length of krill exposed to LL and LD 12:12 may reflect this strategy (Fig. 6a,b). Both explanations require that krill maintain a large lipid depot to drive the enhanced lipid catabolism. The mean total lipid content of all krill at the start of our experiment was 34% of DW. Hagen et al. (1996) and Atkinson et al. (2002), reported mean lipid reserves of 39.2% DW and 44.0% DW in the field at the end of the productive season indicating that our krill had accumulated sufficiently large lipid reserves to allow them to use these stored lipids as a source of energy.

Comparing the trends in lipid utilization in our experiment (conducted from June until Aug) with information from the field, it is notable that krill exposed to LL and LD 12:12 were showing the same trend as animals in the field. In the wild, total lipid data for adult krill exhibit seasonal variations with the highest levels in autumn (April/May) followed by a decrease until early spring (Oct/Nov; see Hagen et al. 2001). This decrease in lipid reserves over the winter months matches the timing when female krill complete their thelycum
Fig. 6. Temporal changes in biochemical composition and body length of krill (this study) related to temporal changes of energy accumulation of krill (Teschke et al. 2007) over the experimental period (12 weeks). Left axis: percentage change in total lipid, total protein and body length of Antarctic krill exposed to (a) continuous light (LL), (b) 12 hours of light and 12 hours of darkness (LD 12:12), and (c) continuous darkness (DD). The points represent a mean of four animals. Right axis: daily carbon ration of Antarctic krill exposed to (a) continuous light (LL), (b) 12 hours of light and 12 hours of darkness (LD 12:12), and (c) continuous darkness (DD) from Teschke et al. (2007). The points represent the mean of three replicate subsamples.
development and most of the females are externally mature by Oct/Nov (Siegel 1988), but still have not started ovary maturation. During the present study, no ovarian growth was observed for female krill showing an accelerated succession of external maturity. Experimental and the field data suggest that this may indicate that the development of external maturation of krill is mainly fuelled by lipid reserves accumulated during the previous productive season, but the subsequent ovary development is fuelled by the spring bloom available in the lead up to the reproductive season.

Overall, the results presented in our current study and those available from the previous study (Teschke et al. 2007) emphasize the role of the Antarctic light regime as an essential cue governing the seasonal cycle of krill physiology and maturity, and highlight the importance of this environmental factor in the life history of krill.

The results indicate that the life-cycle of krill does not generally reflect the annual cycle of food concentration in the environment. That would suggest that krill are able to make use of any improvement in feeding conditions and to transfer these immediately into maintenance of vital functions (growth, reproduction, and metabolism) irrespective of season (light conditions). If so, the timing of growth and reproduction would simply reflect energy availability and maybe keyed to high food concentrations in the water column, typically occurring during the summer phytoplankton blooms, but could also occur during winter in conditions of rich food. Furthermore, reduced metabolism of krill in winter could be caused directly by the scarcity of food at this time of the year and simply reflects a change in ingestion rate, and therefore reduction of metabolism could also exist in summer conditions of sparse food.

We demonstrated that the light conditions that simulated an prolonged summer light regime, coinciding with an enhanced primary production and the main spawning season in the wild, caused increases in physiological parameters of krill such as feeding activity and metabolism (Teschke et al. 2007) and in addition either directly or indirectly accelerate the maturation process of krill (this study). Although all experimental groups received the same amount of food, krill exposed to simulated autumn/spring conditions showed a more consistent increase in physiological parameters and a more subtle development of maturity, while in contrast at no point during this experiment krill exposed to simulated winter light conditions showed a similar response. Even though, the specific mechanisms for these processes have not been fully understood yet, the existing results indicate that beside krill’s
potential for physiological plasticity light conditions in the Antarctic play an important role to adjust the seasonal development of krill to a highly seasonal environment.

Acknowledgements

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Melatonin and its possible role in mediating seasonal metabolic changes of Antarctic krill, *Euphausia superba*

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Abstract

Melatonin, the chief secretory product of the vertebrate pineal gland is suspected to be an ubiquitous molecule principally involved in the transduction of photoperiodic information. Besides vertebrates, melatonin has been detected throughout phylogeny in numerous non-vertebrate taxa. In the present study, the occurrence of melatonin in Antarctic krill *Euphausia superba* and its possible role in mediating seasonal metabolic changes was evaluated. Melatonin was quantified by enzyme linked immunosorbent assay (ELISA) in high performance liquid chromatography (HPLC)-purified extracts of eyestalks and hemolymph of krill sampled in the Lazarev Sea during the Antarctic winter and summer. In addition, oxygen uptake rate and the activity of the metabolic enzyme malate dehydrogenase (MDH) was recorded to assess the metabolic status of krill. Validation of melatonin measurements were carried out on the basis of three different extraction methods with parallel determination of melatonin by ELISA in crude extracts and in HPLC-purified extracts, and after derivatisation of melatonin under alkaline conditions in the presence of hydrogen peroxide (HPLC-PD). A significantly higher respiration rate and MDH activity was found in summer krill than in winter krill indicating that krill was in a state of reduced metabolic activity during winter. However, neither during winter nor during summer there were detectable melatonin concentrations in the visual system or hemolymph of krill. No substantial differences were observed between different extraction methods and quantification procedures. Based on these results, we question the mediating role of melatonin in the control of seasonal metabolic changes in Antarctic krill in particular and its physiological significance in krill in general.
Introduction

Antarctic krill (*Euphausia superba*, hereafter krill) is known as a key species of the Southern Ocean marine ecosystem. Krill has a circumpolar distribution with their main centres of concentration along the continental shelf break and slope, mainly occurring in the upper 200 m of the water column (Marr 1962; Quetin and Ross 1991; Nicol 2006). In general, krill distribution overlaps with that of winter sea ice (Smetacek and Nicol 2005), indicating a close relationship between krill and seasonal sea ice dynamics (Smetacek at al. 1990; Siegel and Loeb 1995; Atkinson et al. 2004). Due to its limitation to higher latitudes and its distribution within the area covered by the seasonal advance and retreat of sea ice, krill experience significant seasonal shifts in photoperiod and light intensity.

It is well accepted that most organisms that inhabit seasonally changing environments have evolved control systems to regulate their physiology by initiating physiological changes due to seasonally significant variables, such as photoperiod or light intensities (Gwinner 1986). However, to date, light as an environmental factor has been given minor consideration as a parameter affecting krill physiological functions. There is now experimental evidence that changes in the environmental light regime have an important effect on physiological parameters of krill. Teschke et al. (2007) observed changes in feeding and metabolic rates of krill related to different simulated Southern Ocean light conditions. Although all experimental groups received the same amount of food, krill exposed to prolonged photoperiod and enhanced light intensity showed an increase in feeding and metabolic activity over the experimental period. In contrast, krill exposed to continuous darkness showed no physiological response. This study indicates that the seasonal course of light conditions in the Antarctic environment is a significant factor that may constitute seasonal (annual) physiological rhythms of krill in the wild.

Seasonal changes of the metabolic status of *E. superba* in the wild have been reported repeatedly. Compared to summer values, several studies demonstrated a reduction in metabolic rate at the onset of winter (Atkinson et al. 2002; Meyer et al. 2002; Cullen et al. 2003) and during winter (Kawaguchi et al. 1986; Quetin and Ross 1991; Torres et al. 1994). Experimental and field data suggest that seasonal changes of the metabolic status of adult krill are the result of fundamental seasonal adaptations in the animal’s physiology irrespective of ambient food levels, triggered and/or driven by the Antarctic light regime. However, up until now the mechanism by which changes in the light regime are transmitted or transduced and the physiological components that are involved remain unclear.
One possible way of analyzing such interaction consists in the identification of substances that are known to play a role in the transduction of light signals. In vertebrates, it is demonstrated that the pineal gland is implicated in conveying photoperiodic information via the daily pattern of melatonin secretion. They have a typical increase in melatonin production during the night, and melatonin is involved in the entrainment of circadian rhythms of behaviour and physiology (Underwood et al. 1987, Reiter 1991). However, it appears that melatonin is an evolutionary conservative molecule with a widespread distribution in the living world. Besides vertebrates, melatonin has been detected throughout phylogeny in numerous non-vertebrate taxa including bacteria, protozoans, macroalgae, vesicular plants, fungi and invertebrates (Vivien-Roels and Pévet 1993, Balzer and Hardeland 1996, Hardeland and Fuhrberg 1996, Hardeland and Poeggeler 2003). The widespread occurrence of melatonin indicates an early evolution and it is assumed that it is principally involved in the transduction of photoperiodic information in living organisms (Vivien-Roels and Pévet 1986). Preliminary melatonin determinations in eyestalks and hemolymph of Antarctic krill showed concentrations of ~1 pg mg$_{tw}^{-1}$ and ~0.2 pg µL$^{-1}$ immunoreactive melatonin, respectively (Teschke et al. 2007) but a distinct pattern of melatonin production and its influence on physiological mechanisms and behaviour in Antarctic krill has not been proven yet.

In general, immunoassays provide rapid and sensitive methods for the quantitative analysis of melatonin in complex sample mixtures, but the high specificity of antibody-antigen binding does not automatically guarantee a valid assay. Safe determinations of melatonin require extra validation, providing comparative determinations with fundamentally different procedures, in addition to appropriate internal and external calibration procedures. Pape and Lüning (2006) developed a highly selective method for the reliable quantification of melatonin based on high performance liquid chromatography (HPLC) purification and subsequent quantification by enzyme linked immunosorbent assay (ELISA). This method allows highly sensitive melatonin determinations in diverse organisms with a low risk of overestimations by false positive results.

The overall aim of the present study was (1) to proof the occurrence of melatonin in Antarctic krill according to the method of Pape and Lüning (2006), (2) to measure melatonin levels and metabolic activities in krill from different seasons to examine melatonin’s possible implications in mediating seasonal metabolic changes of krill, and (3) to validate melatonin determinations by directly comparing different extraction methods and determination procedures. Melatonin was quantified by ELISA in HPLC-purified extracts of eyestalks and hemolymph of krill sampled in the Lazarev Sea during the Antarctic winter and summer.
Additionally, oxygen uptake measurements and the activity of the metabolic enzyme malate dehydrogenase (MDH) were recorded to assess the metabolic status of krill. Comparative melatonin measurements were carried out on the basis of three different extraction methods (ethanol-chloroform, perchloric acid, acetone) with parallel determination of melatonin by ELISA in crude extracts and in HPLC-purified extracts, and after derivatisation of melatonin under alkaline conditions in the presence of hydrogen peroxide (HPLC-PD; precolumn derivatisation).

This study examined the occurrence of melatonin and its possible role in controlling seasonal metabolic functions in Antarctic krill for the first time and thus provides further insights in the mechanism by which changes in the Antarctic light regime affect the physiology of this key species in the Southern Ocean.

Materials and methods

Animals

Live *E. superba* were sampled during a summer (ANT XXIII/2: 19 Nov 2005 to 12 Jan 2006) and winter (ANT XXIII/6: 17 June to 21 Aug 2006) cruise with RV *Polarstern* along parallel meridional transects in the Lazarev Sea, within the scope of the Lazarev Sea krill study (LAKRIS, see Fig. 1). The transects extended from the continental coast into oceanic waters and were located between 6° W and 3° E, and 60° and 70° S. Krill were collected using a RMT8+1 net in the top 200 m of the water column. Immediately after the haul, one part of freshly caught krill were used to (a) measure oxygen uptake rates, and (b) for subsampling hemolymph, which was collected with a syringe from under the carapace, and stored at -80°C until analysis. Another part of freshly caught krill were immediately frozen in liquid nitrogen and were then maintained at -80°C for measurements of MDH activity and melatonin quantification in eyestalks.

Respiration measurements

Oxygen uptake measurements were run in filtered seawater (0.1 µm pore size). Krill were incubated for 24 h individually in 2.5 L bottles. One bottle of the same volume without krill was used as control. The krill were rinsed and added to the incubation bottles, which were then topped-up and sealed with parafilm. At the end of the incubation time sub-sampling was done by inserting a glass tube, and siphoning the mixed contents of each bottle into 50 mL Winkler bottles, according to Atkinson et al. (2002). Three replicates were filled for each
experimental bottle. Oxygen concentrations were measured after immediate fixation for Winkler titrations as described in Meyer et al. (2002), using a 702 SM Titrino (Metrohm). The decrease in oxygen concentration for all experiments was < 20%.

Fig. 1. Location of the LAKRIS (Lazarev Sea krill study) survey area.

MDH activity

MDH activity (E.C. 1.1.1.37) was measured in the fifth abdominal segment which was dissected on ice. The segment was placed into pre-weighted microtubes and wet weight was analyzed by using a microbalance. Extracts of segment tissues were prepared in 500 µL deionised water. Homogenisation was performed on ice with a Bio-Vortexer™ for 10 s, followed by sonicating with an ultrasonic cell disrupter (Sonopuls, Bandelin) with three bursts of 10 s (30% duty cycle, 30% output). The homogenates were then centrifuged for 15 min at 2000 g and 4°C. The supernatants were transferred into new reaction cups and MDH activity was determined as follows: 810 µL reaction buffer (0.1 mol L⁻¹ potassium phosphate, pH 7.0), 30 µL nicotinamide adenine dinucleotide, reduced form (NADH, 7 mmol L⁻¹ in distilled water) and 30 µL sample were mixed in a semi-microcuvette. After 2 min of pre-incubation at 30°C the reaction was initiated by adding 30 µL oxaloacetate (12 mmol L⁻¹ in distilled water). The change in absorbance at 340 nm was recorded for another 3 min. The activity was expressed as µmol min⁻¹ g⁻¹ fw (µmol min⁻¹ g⁻¹ fw⁻¹) using the extinction coefficient for NADH ε₃₄₀ = 6.22 L mmol⁻¹ cm⁻¹.
Melatonin quantification in eyestalks and hemolymph

Melatonin was quantified in eyestalks and hemolymph of krill by ELISA in HPLC-purified extracts (Fig. 2).

**Fig. 2.** Processing scheme for the quantification of melatonin in eyestalks and hemolymph of Antarctic krill. Parallel measurements with additions of synthetic melatonin prior to the extraction were made for determinations of recovery rates. Melatonin was quantified by enzyme-linked immunosorbent assay (ELISA) in high-performance liquid chromatography (HPLC)-purified extracts after ethanol-chloroform extraction.

*Sample preparation and extraction*—All processing was done in dim light (<0.1 µmol photons · m⁻² · s⁻¹) in the laboratory. 50 mg eyestalks (~ten pairs) per extraction were placed into pre-weighted microtubes and wet weight was analysed by using a microbalance. Homogenisation was performed on ice with a Bio-Vortexer™ for 10 s in 300 µL ethanol (EtOH); 1500 µL deionised water was added and the homogenate was then sonicated with an ultrasonic cell disrupter with three bursts of 10 s (30% duty cycle, 30% output). Melatonin was extracted with 15 mL chloroform according to Tilden et al. (1997) with gentle shaking at 20°C for 30 min. The chloroform was then transferred to new test tubes and evaporated under a constant stream of nitrogen. Before injection to the HPLC system, the residue was reconstituted in 140 µL HPLC eluent (see below) for 30 min with intermittent mixing. 100 µL of pooled hemolymph per extraction was placed into test tubes and was mixed with 1500 µL deionised water and 300 µL EtOH. Then 5 mL of n-hexane was added and the tubes were gently shaken at 20°C for 10 min. After centrifugation at 2000 g and 4°C for 5 min the
aqueous phase was separated and placed into 50 mL-PE tubes. Melatonin was then extracted as described for eyestalks.

For determinations of the recovery rates, 50 µl of the deionised water was substituted by melatonin spiked water (0.1 pg · µl⁻¹).

**HPLC purification**—Extracts were reconstituted in eluent (12.66 g citric acid, 2.21 g NaH₂PO₄, 2.21 g Na₂HPO₄, 1.0 g octanesulfonic acid, 33 mg EDTA and 185.5 ml methanol adjusted with water to 1 L total volume, pH 5.5) for 30 min and were then centrifuged at 20,000 g for 5 min at 4°C. The supernatants were injected to the HPLC system. A fluorescence detector (RF2000, Dionex) was used for determination of melatonin retention time. For a detailed description of the HPLC system see Pape and Lüning (2006). For melatonin purification a 1-min fraction (1 mL) containing melatonin was collected manually with a Pyrex glass tube at the outlet of the fluorescence detector (the time window started 0.4 min before melatonin retention time and ended 0.6 min thereafter). Immediately, 2 ml chloroform were added and vortexed for 5 sec at maximum speed for liquid phase extraction of melatonin from the mobile phase. Samples were then stored overnight in darkness at 6°C. Next day, after centrifugation at 2000 g for 2 min (10°C), the lower chloroform phase was transferred to a new glass tube and evaporated under a stream of nitrogen. The residue was dissolved in 250 µl reconstitution buffer (EK-DSM-RB) for 30 min and then stored at -80°C until ELISA measurement.

To determine recovery rates and demonstrate accuracy of the purification step, five 1-min fractions (1 mL each) were collected from unspiked and melatonin spiked extracts. Two 1-min fractions before the melatonin retention time (-2, -1), the 1-min fraction containing melatonin (M), and two 1-min fractions after the melatonin retention time (+1, +2). The fractions were then processed as described above.

**Enzyme-linked immunosorbent assay**—A commercially available ELISA kit (EK-DSM; Buehlmann) was used for immunological melatonin determinations. This ELISA kit was developed for direct saliva melatonin measurements and is characterized by a high sensitivity (0.1 pg · well⁻¹) and low cross reactivity with structurally related substances.

The ELISA was used according to the manufacturer's instruction except for using self-made melatonin standard ranging from 0.05 to 5 pg · well⁻¹. Melatonin standard (100 pg · µl⁻¹ in methanol) was diluted with methanol to a concentration of 0.025 pg · µl⁻¹ and 5, 15, 50, 100, 200 or 500 µL were pipetted into reaction tubes. After evaporation of the methanol under
a stream of nitrogen, 250 µl reconstitution buffer was added to each tube and the melatonin was allowed to dissolve for 30 min. Towards the end of the assay, after addition of the stop solution, the optical density at 450 nm was measured by using a micro plate reader (Synergy HT, BIO-TEK). Calculations of melatonin concentrations from ELISA data were performed using the software Fig.P (Biosoft; vers. 2.98).

Comparative melatonin measurements

To validate the quantification of melatonin, homogenous krill material was used to compare three different extraction methods with parallel testing of three different melatonin determination procedures (Fig. 3).

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**Fig. 3.** Processing scheme for the comparative quantification of melatonin in homogenous eyestalk material of Antarctic krill using different extraction methods and purification steps. Parallel measurements with additions of synthetic melatonin prior to the extraction were made for determinations of recovery rates. Melatonin was quantified by enzyme-linked immunosorbent assay (ELISA) in crude extracts, high-performance liquid chromatography (HPLC)-purified extracts and after derivatization of melatonin under alkaline conditions in the presence of hydrogen peroxide (HPLC-PD). The results can directly be compared to estimate the efficiency of the methods, as homogenous material was used.

The different extraction methods were based on ethanol-chloroform (Tilden et al. 1997), perchloric acid (PCA, Poeggeler and Hardeland 1994), and acetone (Balzer et al. 1997). PCA extraction was used in combination with C₁₈ solid phase extraction (SPE) for purification and
Melatonin and its possible role…

Sample preparation and extraction—1.5 g frozen eyestalks were added to a mortar and preground in liquid nitrogen to a homogenous powder. The powder was then stored at -80°C for a maximum of four weeks before extraction. All processing was done in dim light (<0.1 µmol photons · m⁻² · s⁻¹). For determinations of the recovery rates, 40 µl of the primary solvent were substituted by melatonin spiked solvent (1 pg · µl⁻¹).

For the ethanol-chloroform method, 300 µL EtOH and 1500 µL deionised water were added to 80 mg eyestalk material in a test tube. The mixture was then sonicated with an ultrasonic cell disrupter with three bursts of 10 s (30% duty cycle, 30% output), and melatonin extraction was performed as described above. The chloroform phase was then transferred to new test tubes and evaporated under a constant stream of nitrogen. The residue was dissolved in 1000 µL methanol (MeOH) and was separated into an aliquot of 10% (100 µL) for direct melatonin ELISA determination and 90% (900 µL) for HPLC-purification. Both aliquots were then again evaporated under a stream of nitrogen. The residue for the direct ELISA was dissolved in 270 µl reconstitution buffer (EK-DSM-RB) for 30 min, while the residue for HPLC-purification was reconstituted in 140 µL HPLC eluent for 30 min with intermittent mixing.

For the PCA method, 80 mg eyestalk material was mixed with 1000 µL PCA (0.4 mol L⁻¹) in a test tube and then sonicated with an ultrasonic cell disrupter with three bursts of 10 s. After incubation for 30 min at 4°C the mixture was centrifuged at 20,000 g for 10 min at 4°C. One-ml solid phase extraction columns (Bakerbond spe Octadecyl (C₁₈), 100 mg, 40 µm) were used for further purification and concentration of PCA extracts. After conditioning with 2x 1 ml methanol and 2x 1 ml water, 1000 µL extract was loaded onto the column and washed with 2x 1 ml water:methanol (90:10) and 1 ml n-hexane. After elution with 1 ml methanol the organic solvent was separated into two aliquots (10%, 90%), evaporated under a stream of nitrogen and redissolved in 270 µl reconstitution buffer and 140 µL HPLC eluent for direct ELISA measurement and HPLC-purification, respectively.

For the acetone method, 80 mg eyestalk material was mixed with 1000 µL cold acetone in a test tube and then sonicated with an ultrasonic cell disrupter with three bursts of 10 s. After centrifuging the mixture at 20,000 g for 10 min at 10°C the supernatant was transferred to a
clean glass tube and then separated into two aliquots for direct ELISA measurement and HPLC-purification (10%, 90%). After dilution with an equal volume of deionized water to ensure freezing at -80°C the aliquots were lyophilized. Lyophilisates were then dissolved in 270 μL reconstitution buffer and 140 μL HPLC eluent as described above.

All aliquots dissolved in reconstitution buffer were stored at -80°C for a maximum of 4 weeks until ELISA measurement. Aliquots reconstituted in HPLC eluent were centrifuged (see above) and injected to the HPLC system. A 1-min fraction (1 mL) containing melatonin was collected and extracted as described before.

For ELISA determinations of purified extracts, 10% of the chloroform phase of a single HPLC run was evaporated under a constant stream of nitrogen and dissolved in 270 μl reconstitution buffer for 30 min and then stored at -80°C until analyzed. The remaining chloroform was then evaporated for HPLC-PD determination (see below).

**HPLC-PD**— Melatonin was measured in parallel by a method based on derivatization of melatonin to N-[(6-methoxy-4-oxo-1,4-dihydroquinoline-3-yl)methyl]acetamid (6-MOQMA) under alkaline conditions in the presence of hydrogen peroxide (Inuma et al. 1999; Tomita et al. 2003; Hamase et al. 2004). The product was quantified fluorometrically (λ<sub>ex</sub> / λ<sub>em</sub> = 245 / 380 nm) by the same HPLC system that was used for purification of the extracts. The residue from HPLC purified samples was redissolved in 120 μl H<sub>2</sub>O. After 30 min with intermittent vortexing, 12 μl of Na<sub>2</sub>CO<sub>3</sub> (1 mol L<sup>-1</sup>) and 12 μl H<sub>2</sub>O<sub>2</sub> (0.05 mol L<sup>-1</sup>) were added, capped and incubated in a water bath at 93°C for 90 min. Samples were allowed to cool down before injection to the HPLC system. Melatonin concentration was calculated on basis of integrated peak area.

Statistical analysis

All tests were performed with the computer program SigmaStat 3.00 (SPSS). An unpaired t-test was used to perform summer-winter comparisons of krill respiration rates, MDH activities, melatonin concentrations in eye stalks and hemolymph, and to compare melatonin values of krill from the summer and winter, processed with three different extraction methods and quantified in parallel with three quantification procedures. Results were considered significantly different at a p value of < 0.05. Data were expressed as mean ± standard deviation (SD).
Results

Respiration rates, MDH activities, and melatonin contents in eye stalks and hemolymph of krill sampled in the Lazarev Sea during Antarctic winter and summer are shown in Table 1. A significantly higher mean respiration rate was found in summer krill (0.62 µl · mg dw⁻¹ h⁻¹), than in winter krill (0.22 µl · mg dw⁻¹ h⁻¹). A significant difference was also found in the mean activity of the metabolic key enzyme MDH between summer (78.8 U · g fw⁻¹) and winter krill (32.9 U · g fw⁻¹). Respiration rate and MDH activity indicate metabolic changes at overall and molecular levels.

No significant difference in the mean melatonin content was found between summer and winter krill (processed according to Fig. 2). In eye stalks of krill, the mean melatonin concentration was 6.1 ± 1.4 pg · g fw⁻¹ during summer and 6.3 ± 3.1 pg · g fw⁻¹ during winter, whereas melatonin was found in hemolymph with mean values of 2.4 ± 0.3 pg · ml⁻¹ in summer and 2.9 ± 0.5 pg · ml⁻¹ in winter. However, despite the high sensitivity of the ELISA kit (0.1 pg · well⁻¹), the levels of melatonin immunoreactivity found in HPLC-purified extracts of eye stalks and hemolymph were below the detection limit of the method corresponding to 7.6 pg · g fw⁻¹ and 3.8 pg · ml⁻¹, respectively.

Table 1. Respiration rate, MDH activity, and melatonin content in eye stalks and hemolymph of *E. superba* from the Lazarev Sea during summer and winter. Melatonin values were processed according to Fig. 2. Values represents means (± SD), (n).

<table>
<thead>
<tr>
<th>Season</th>
<th>Respiration (µl · mg dw⁻¹ h⁻¹)</th>
<th>MDH (U · g fw⁻¹)</th>
<th>Melatonin in eye stalks (pg · g fw⁻¹)</th>
<th>Melatonin in hemolymph (pg · ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer</td>
<td>0.62 (±0.18) * (9)</td>
<td>78.8 (±34.5) * (8)</td>
<td>6.1 (±1.4) (8)</td>
<td>2.4 (±0.3) (3)</td>
</tr>
<tr>
<td>Winter</td>
<td>0.22 (±0.06) * (23)</td>
<td>32.9 (±11.4) * (8)</td>
<td>6.3 (±3.1) (8)</td>
<td>2.9 (±0.5) (3)</td>
</tr>
</tbody>
</table>

*indicates significant differences between summer and winter (p < 0.05).

The HPLC system could not be used for direct quantification of melatonin in extracts of eye stalks and hemolymph because of numerous substances giving a fluorescence signal with a similar retention time than that of the melatonin peak. However, the system was used for purification of the extracts by manually collecting the melatonin fraction and subsequent liquid phase extraction with chloroform (see Fig. 4). No destruction of melatonin during passage through the fluorescence detector’s UV light was noticed.
Fig. 4. Representative chromatogram of krill hemolymph in the preparative HPLC system after extraction with ethanol-chloroform. For melatonin purification a 1-min fraction (1 mL) containing melatonin was collected manually with a Pyrex glass tube at the outlet of the fluorescence detector and processed as described. The lower HPLC run shows melatonin standard (10 pg in 100 µl injection volume). Melatonin retention time was 8.3-8.4 min. To determine recovery rates and demonstrate accuracy of the purification step, five 1-min fractions (1 mL each) were collected from unspiked extracts and after synthetic melatonin was added (see Fig. 5). Two 1-min fractions before the melatonin retention time. A 1-min fraction containing melatonin and two 1-min fractions after the melatonin retention time, $\lambda_{ex} / \lambda_{em} = 285 / 360$ nm.

When synthetic melatonin was added at a concentration of 0.1 pg · mg $\text{fw}^{-1}$ for eye stalks and 0.05 pg · $\text{µl}^{-1}$ for hemolymph and quantified thereafter with ELISA, it was always found exclusively in the melatonin fraction (103.3 ± 10.5 pg · g $\text{fw}^{-1}$ and 21.9 ± 4.2 pg · $\text{ml}^{-1}$), detected at a recovery of 103% and 44%, respectively (Fig. 5). In contrast, when no melatonin was added, levels of melatonin immunoreactivity were found almost exclusively below the detection limit of the method (see above) and no differences between the collected fractions were observed. Values in eye stalk extracts ranged between 5.2 ± 3.3 pg · g $\text{fw}^{-1}$ in fraction -2 and 6.4 ± 1.4 pg · g $\text{fw}^{-1}$ in fraction -1. For hemolymph extracts, melatonin immunoreactivity were between 2.0 ± 1.0 pg · ml$^{-1}$ in fraction -1 and 9.1 ± 3.7 pg · ml$^{-1}$ in fraction +1.

To validate the quantification of melatonin, homogenous krill material from summer and winter was used to compare three different extraction methods with parallel testing of three different melatonin determination procedures (Table 2). For all three extraction methods, Melatonin immunoreactivity for summer and winter determined by ELISA in crude extracts ranged between 20.8 and 48.6 pg · g $\text{fw}^{-1}$. Except after PCA and acetone extraction of summer samples and ethanol-chloroform extraction of winter samples, melatonin levels were below the detection limit of this method (33.3 pg · g $\text{fw}^{-1}$). Melatonin immunoreactivity in HPLC-purified extracts were almost exclusively found below those of crude extracts ranging between 3.7 and 36.3 pg · g $\text{fw}^{-1}$. Corresponding to the sensitivity of the HPLC-ELISA method
(52.6 pg · g\(^{-1}\) fw), all values found were below the detection limit of the method. No melatonin was found in summer and winter krill with the HPLC-PD method for all three extraction methods.

**Fig. 5.** Recovery of synthetic melatonin that was added at the beginning of the extraction procedure at a concentration of 0.1 pg · mg\(^{-1}\) fw for (A) eye stalks and 0.05 pg · µl\(^{-1}\) for (B) hemolymph. After extracts were injected to the preparative HPLC system, five 1-min fractions (1 mL each; two 1-min fractions before the melatonin retention time, one 1-min fraction containing melatonin, and two 1-min fractions after the melatonin retention time) were collected, processed as described, and thereafter melatonin was quantified with ELISA. The bars show means ± SD, (n=3).
Table 2. Melatonin content of *E. superba* from the Lazarev Sea during summer and winter. Samples were processed with three extraction methods (see Fig. 3) and melatonin was quantified in parallel with ELISA in crude (ELISA direct) and HPLC-purified (HPLC-ELISA) extracts, and with HPLC after derivatization of the melatonin (HPLC-PD). For determination of recovery 40 pg were added before extraction. Value represents means (± SD), (*n* = 3).

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Quantification</th>
<th>Summer</th>
<th>Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg · g fw⁻¹</td>
<td>pg · g fw⁻¹ added</td>
<td>pg · g fw⁻¹ found</td>
</tr>
<tr>
<td>PCA</td>
<td>ELISA direct</td>
<td>48.6 (± 17.4)</td>
<td>456.3 (± 15.5)</td>
</tr>
<tr>
<td></td>
<td>HPLC-ELISA</td>
<td>36.3 (± 11.4)</td>
<td>456.3 (± 15.5)</td>
</tr>
<tr>
<td></td>
<td>HPLC-PD</td>
<td>0</td>
<td>456.3 (± 15.5)</td>
</tr>
<tr>
<td>Acetone</td>
<td>ELISA direct</td>
<td>34.7 (± 9.9)</td>
<td>487.3 (± 8.8)</td>
</tr>
<tr>
<td></td>
<td>HPLC-ELISA</td>
<td>30.8 (± 29.5)</td>
<td>487.3 (± 8.8)</td>
</tr>
<tr>
<td></td>
<td>HPLC-PD</td>
<td>0</td>
<td>487.3 (± 8.8)</td>
</tr>
<tr>
<td>EtOH-Chlo.</td>
<td>ELISA direct</td>
<td>26.5 (± 2.3)</td>
<td>479.0 (± 8.8)</td>
</tr>
<tr>
<td></td>
<td>HPLC-ELISA</td>
<td>23.0 (± 1.1)</td>
<td>479.0 (± 8.8)</td>
</tr>
<tr>
<td></td>
<td>HPLC-PD</td>
<td>0</td>
<td>479.0 (± 8.8)</td>
</tr>
</tbody>
</table>

No significant differences were observed when comparing melatonin values of one quantification procedure (within a season), processed with different extraction methods, nor when comparing values of one quantification procedure (processed with the same extraction method) between seasons. However, when melatonin was added at the beginning of the extraction procedure the recovery was always between 37% and 92%. Lowest recovery rates were found for acetone extraction of summer samples (37-42%) and for PCA extraction of winter samples (45-54%).

**Discussion**

*E. superba* collected during the Antarctic winter and summer exhibited seasonal metabolic changes that indicate that they were in a state of reduced metabolic activity during winter. Respiration rates were reduced relative to summer, and there were also very low activities of
the metabolic key enzyme MDH. However, there was no indication of a mediating role of melatonin. Neither during winter nor during summer there were detectable melatonin concentrations in the visual system or hemolymph of krill.

The metabolic status of krill is reported to vary seasonally with high rates in summer merging into low or reduced rates in winter followed by a subsequent increase in metabolism from winter to summer. Metabolic proxies at molecular (Meyer et al. 2002; Cullen et al. 2003) and overall levels (Kawaguchi et al. 1986; Atkinson et al. 2002) have given indications for seasonal variation in the metabolic status in krill. It was concluded that the reduction in metabolic rates seems to be the major physiological response to the Antarctic winter, when most of the environment is covered by ice, and food in the water is extremely scarce (Kawaguchi et al. 1986; Quetin and Ross 1991; Torres et al. 1994). The results from this study agree with the finding of the previous studies. Significantly higher oxygen consumption rates and MDH activities in summer krill compared to winter krill reflect the annual rhythm of metabolic activity in krill. MDH is a metabolic enzyme catalyzing reactions in the citric acid cycle and energy metabolism. Activity of MDH shows high positive correlation with oxygen consumption rates and is considered as an effective molecular proxy for metabolic rates (Donnelly et al. 2004), while oxygen consumption rates are determined to assess the overall metabolic level.

Gathering these observations, it makes sense that krill would evolve physiological mechanisms that allows it to allocate metabolic capacity to the times of the year when food is abundant, while switching to an energy-saving mode in winter when food is scarce. In general, annual biological rhythms are most clearly expressed in organisms inhabiting arctic zones, where seasonal differences in environmental conditions are most pronounced (Gwinner 1986). The critical questions become: which environmental factor is involved in the control of this annual rhythm of metabolic and/or physiological activity in krill, and how is such influence characterized.

Atkinson et al. (2002) demonstrated that there is a fundamental seasonal transition in krill physiology, irrespective of ambient food levels that indicate that a reduced metabolism of krill in winter may not be caused simply and directly by the scarcity of food at this time of the year. Teschke et al. (2007) showed that different simulated light regimes affect feeding and metabolism of krill irrespective of food supply and thus underlined the important role of the Antarctic light cycle in the control of physiological parameters of krill. Altogether, these studies indicate that seasonal differences in the metabolic status of krill are caused by an
annual rhythm that enables it to adapt to the extreme seasonal changes in food availability between the Antarctic summer and winter and that is keyed to the seasonal course of light in the Antarctic ecosystem.

Although so far there is no experimental insight in the specific effects of light conditions on physiological functions in krill, it is likely that the decisive cue in the control of such annual rhythm in krill is the seasonal gradual change of day length, photoperiod. In the evolution of control systems of numerous plants and animals the annual cycle in photoperiod has become the major source of predictive environmental information in the control of a variety of seasonal activities (Gwinner 1986; Brandstätter 2003; Schultz et al. 2003). The results presented by Teschke et al. (2007) showed a relatively rapid and direct response of the measured parameters to different light regimes. Assuming that differences in photoperiod treatment have caused the physiological changes, this may indicate that seasonal changes in the physiological status of krill are induced directly by the natural annual course of photoperiod. However, there is also a possibility that the annual rhythm of metabolism in krill is independent of direct control, representing an endogenous circannual rhythm which is synchronized with the natural year by the annual course of photoperiod. In any case, however, whether the annual course of photoperiod provides a direct, causal stimulus that adjusts the metabolic status at the right time in the year, or constitutes a Zeitgeber for the endogenous rhythmicity, krill needs a photoperiodic response system which enables it to measure seasonal changes in day length.

The indoleamine melatonin was hypothesized to be involved in the transduction of photoperiodic information in krill, after preliminary melatonin determinations in eyestalks and hemolymph showed concentrations of \(~1 \text{ pg mg}_{\text{fw}}^{-1}\) and \(~0.2 \text{ pg } \mu \text{L}^{-1}\) immunoreactive melatonin, respectively (Teschke et al. 2007). In this hypothesis a seasonal changing level of melatonin secretion is thought to mediate the effect of photoperiodic changes for the organization of an annual or circannual rhythm of metabolic activity in krill. Hence, melatonin secretion encodes the photoperiodic information directly or in combination with an endogenous oscillator which may be acting “up-line” or “down-line” from the information provided by the melatonin signal.

To date, the presence of melatonin in crustaceans is well documented and its related enzyme, \(N\)-acetyltransferase (NAT), is found within the visual system of several species. Crustacean melatonin is not necessarily circadian, and if so not always with a nocturnal maximum, but has also been shown to exhibit important seasonal changes. The shore crab \(Carcinus maenas\) for example, showed no diurnal variation but seasonal differences between
May (65 pg mg\(^{-1}\)) and November (6.5 pg mg\(^{-1}\)) (Vivien-Roels and Pévet 1986). The intertidal fiddler crab *Uca pugilator* may have a tide-associated melatonin cycle with increased levels in the eyestalks during the day (photophase) (Tilden et al. 1997; Tilden et al. 2001). The crayfish *Procambarus clarkii* showed high melatonin levels during photophase (Agapito et al. 1995) and in another study high levels during the night (scotophase) (Balzer et al. 1997). These studies indicate an important role of melatonin in crustacean physiology and, compared to vertebrates, a greater variability in melatonin production. However, during this study neither in the visual system nor in the hemolymph of krill detectable melatonin concentrations were observed in comparison of different seasons. This indicates that there was no seasonal difference in the quantity of melatonin secretion which was hypothesized to mediate seasonal metabolic changes in krill. For comparison of melatonin concentration between seasons, krill was sampled routinely during nighttime and during daytime, to ensure that possible night/day changes were recorded. However, neither in night-samples nor in day-samples the occurrence of melatonin was observed during the course of the study (results not shown). Consequently, we can also eliminate the possibility that a daily changing profile of melatonin secretion conveys photoperiodic information for the organization of a metabolic seasonal rhythm in krill, as it is known for several mammalian species (Arendt 1998; Oster et al. 2002; Lincoln 2006). In mammals, melatonin is secreted with a circadian rhythm and a typical increase in melatonin production during the night. The duration of secretion thus reflects the duration of the night, resulting in long melatonin signals in winter with the reverse in summer (Goldmann 2001). Melatonin signal duration is then deciphered by specialized melatonin-target cells that control the physiological response (Lincoln et al. 2003).

The results from this study contradict preliminary melatonin determinations in eyestalks and hemolymph of krill (see above). Given the fact that these observations were conducted on the basis of ELISA measurements in crude extracts of krill eyestalks and hemolymph it is possible that melatonin levels were overestimated by false positive results. The chemical complexity of such extracts can interfere with ELISA determinations giving false positive results, due to cross reactivity with antibodies or non-specific inhibition (Pengelly 1985). On the other hand, because of melatonin’s properties as a radical scavenger, the indoleamine is readily degraded by reactive oxygen species that can be generated during extraction procedures (Hardeland and Poeggeler 2003). For these reasons, comparative melatonin measurements in this study were carried out on the basis of three different extraction methods with parallel determination of melatonin by three different procedures (see material and methods). No substantial differences were observed between different extraction methods.
(Table 2, first column). If the endogenous melatonin would have been destroyed within the tissue before entering the primary solvent, or if much destruction was occurring during the first steps of the extraction process due to solvent properties one would expect higher variability between the examined extraction methods. Also, no substantial differences were observed between different quantification procedures (Table 2, second column). Melatonin immunoreactivity in crude extracts were found to be slightly higher than those of HPLC-purified extracts and may indicate interference of certain substances with the ELISA but cannot explain the observed differences between melatonin levels of the preliminary determinations in krill and this study. This sort of discrepancy may be due to the quality of antibodies used in the melatonin assays. The phenomenon of substantial inconsistent results between different assays due to the quality of antibodies was previously described (Arendt 1985). To avoid false positive results by cross reactivity or non-specific inhibition in the ELISA, and to resolve the melatonin derivative peak after HPLC-PD crude extracts in this study were previously purified by preparative HPLC. The combination of HPLC purification and subsequent determination by ELISA and HPLC-PD allow highly sensitive melatonin determinations (see Pape and Lüning 2006). Overall, this study indicates that Antarctic krill does not exhibit any melatonin secretion neither on a diurnal nor on a seasonal basis which then may used to time daily and/or seasonal functions.

Given the observation that the proposed photoperiodic response system in krill is not based on melatonin as a transducer of photoperiodic information, it is unclear at this stage how photoperiodic time measurement is generally realized in krill. There is now some evidence that organisms using the tools of the circadian system to sense changes in daylength and to mediate the photoperiodic response (Oster et al. 2002; Schultz et al. 2003). Principally, three main models for photoperiodic time measurement in organisms have been proposed. The first model, however, argues without the involvement of a circadian system (“hourglass” model) and assumes that a critical night length (or day length) triggers a photoinductive program (Lees 1973). The two other models assume a role of a circadian system and are further classified into the external and internal coincidence model. In the first, light has a dual role: it entrains a circadian rhythm (e.g. production of a photosensitive product) and also acts as a photoperiodic stimulus (Bünning 1936; Davis 2002). In the second model, light’s only role is the entrainment of multiple circadian rhythms (Pittendrigh et al. 1984; Davis 2002). These general models of photoperiodic time measurement can propose mechanisms both for the generation of an annual rhythm of metabolic activity in krill and the synchronization of an endogenous circannual rhythm of metabolic activity in krill to the external annual cycle of
photoperiod. Further experimental studies on krill are required to allow us to build a picture of the mechanism by which the Antarctic light regime affects the physiology of this key species in the Southern Ocean.

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CONCLUSIONS

This thesis evaluates the influence of seasonal light conditions on the physiology of Antarctic krill. The investigations focus on both the effect of different light regimes on certain life-cycle parameters of krill and the occurrence of melatonin in krill and its possible role in the transduction of light information.

With respect to the complex life cycle of krill that has evolved to exploit a highly seasonal environment, it is crucial to understand krill adaptations to its environment and the forces that determine it. Reduction of metabolic and feeding activity of krill during the Antarctic winter is suspected to be an overwintering strategy to survive the times when food is scarce (Kawaguchi et al. 1986; Quetin and Ross 1991; Torres et al. 1994). The onset and timing of krill maturation is important for the coincidence of the reproductive period with times of substantial food supply, and guarantees the reproductive success of krill (Ross and Quetin 2000). However, current uncertainties exist regarding the mechanisms causing synchronization between the seasonal development of krill and the seasonal cycles of environmental features. Krill experience significant seasonal shifts in food availability and light conditions. While previous studies demonstrated that annual changes in the physiological status and maturity development of krill appeared to be not primarily driven by food supply (Thomas and Ikeda 1987; Atkinson et al. 2002; Kawaguchi et al. 2006), it is suggested that changes in the Southern Ocean light regime have an important effect on physiological parameters of krill. In order to address these issues, krill were exposed to laboratory simulations of different Southern Ocean light regimes (summer, autumn/spring, winter) and the effect on feeding and metabolic activity (manuscript I) as well as on the development of sexual maturity (manuscript II) is investigated.

INFLUENCE OF SEASONAL LIGHT CONDITIONS ON THE PHYSIOLOGY OF KRILL

It could be demonstrated that seasonal changes in the environmental light regime have an important effect on feeding and metabolic activity of krill (manuscript I). The results indicate that, irrespective of food supply, the level of feeding and metabolic activity in krill correlates with the degree of prevailing light conditions. Thus, a summer light regime of prolonged photoperiod and enhanced light intensity implicates higher feeding and metabolic activity than a more attenuated light regime of autumn/spring conditions, or even more, of a winter light regime. Consequently, light conditions not only affect the life-cycle of krill indirectly
through regulating the seasonal pattern of primary production but also have a direct effect and provide an essential cue that enables krill to adapt to seasonal variations in food supply. This mechanism makes sense ecologically, and allows krill to allocate metabolic capacity to the times of the year when food is abundant, while saving energy during winter when food is scarce. In this way, there is evidence that reduced values of feeding and metabolic activity of krill during winter are not caused directly by the scarcity of food at this time of the year and simply reflect a change in ingestion rate, but represent an inherent adaptational overwintering strategy triggered by the Southern Ocean light regime.

The findings from manuscript I are considered for the investigations made in manuscript II. Assuming that environmental light conditions are linked to the seasonal life cycle of krill, differences in the succession of female and male krill external sexual maturity stages are observed on the experimental animals.

It could be demonstrated that changes in the environmental light regime also have an important effect on the maturity development of krill. The results indicate that the succession of female and male krill maturity stages is accelerated by light conditions of prolonged photoperiod and enhanced light intensity. This indicates that the development of maturity does not simply reflect energy availability, and is not directly keyed to high food concentrations in the water column, typically occurring during the summer phytoplankton blooms. Krill reproduction requires a long period of maturity development and cannot be initiated immediately when environmental conditions become favourable. It is crucial that the development of external sexual characteristics of krill start in advance at times when food conditions are not optimal to ensure the coincidence of abundant food resources and the maximum food requirement (for adults and the offspring). In this way, there is evidence that light conditions in the Southern Ocean play an important role to adjust the onset and timing of krill maturation in the wild and thus in the adaptation of krill reproduction to a highly seasonal environment.

The Antarctic krill has evolved a life history that is obviously highly successful at exploiting its seasonally variable environment. As a prerequisite, krill is thought to show a large potential for physiological plasticity using favourable conditions immediately for vital functions such as growth, reproduction and metabolism (Buchholz and Saborowski 2000). However, the experimental data of manuscript I and II indicate that annual rhythms in life-cycle parameters of krill do not generally reflect the annual cycle of food concentration in the environment. That would suggest that krill is able to make use of any improvement in feeding conditions and to transfer these immediately into maintenance of vital functions irrespective
of season (light conditions). Instead, the results indicate that, beside krill’s potential for physiological plasticity, these rhythms are more the result of fundamental seasonal adaptations in the animal physiology. This emphasizes the role of the Southern Ocean light regime as an essential cue governing the seasonal cycle of krill physiology. The annual course of light conditions provides reliable and predictive information for the control of physiological processes in krill. Altogether, the findings give evidence that krill have evolved control mechanisms to anticipate seasonal variations in light conditions and thereby to anticipate the succession of the season. This enables krill to regulate its physiology due to the pronounced seasonal differences in environmental conditions.

The findings from manuscript I and II also give indications regarding the specific response mechanisms to differences in light conditions. In general, annual cycles of physiology or behaviour may be controlled directly by the annual cycle of light, or may be pre-programmed as endogenous circannual rhythms (Gwinner 1986). The latter implicates that such rhythms are independent of direct control of light conditions, and are driven by an endogenous clock which is synchronized with the natural year. Previous studies on the maturity cycle of krill showed that the cyclic maturation process represents an endogenous rhythm, independent of direct control by factors such as food or light (Thomas and Ikeda 1987; Kawaguchi et al. 2006). Together with the results from manuscript II, this indicates that the annual pattern of krill maturity development in the field most likely represents a circannual rhythm driven by an endogenous clock which is synchronized with the natural year by the annual course of light conditions. Light then act as a synchronizer (Zeitgeber) and thus link the endogenous maturity cycle to the natural year. Considering the effects of different simulated light regimes on feeding and metabolic activity of krill (manuscript I), there is a possibility that light may also affect the maturation cycle through regulating seasonal patterns of feeding activity and energy accumulation. Hence, an increase of energy accumulation may then have accelerated the succession of krill maturity. Whether seasonal changes of feeding and metabolic activity of krill also reflect a circannual rhythm remains unclear at this stage. The results presented in manuscript I show a relatively rapid and direct response of the measured parameters to different light regimes. This may indicate that seasonal changes of feeding and metabolic activity of krill are affected directly by the natural annual course of light conditions which then consequently provide a direct and causal stimulus.

In order to simulate the summer and autumn/spring conditions most accurately, we adjusted the light regimes to different photoperiods and light intensities. Although so far,
there is no experimental insight into the specific effects of light conditions on physiological functions in krill, it is likely that the decisive cue in the control of annual rhythms in krill is the seasonal gradual change of day length, photoperiod. In the evolution of control systems of numerous plants and animals the annual cycle in photoperiod has become the major source of predictive environmental information in the control of a variety of seasonal activities (Gwinner 1986; Brandstätter 2003; Schultz et al. 2003).

Conclusions of manuscripts I and II:

- Annual rhythms in feeding and metabolic activity of krill do not generally reflect the annual cycle of food concentration in the environment.

- The Southern Ocean light regime is an essential cue governing the annual cycle of krill physiology:
  
  ⇒ The reduction of feeding and metabolic activity of krill during winter represent an inherent adaptational overwintering strategy triggered by the Southern Ocean light regime.

  ⇒ Light conditions play an important role in adjusting the onset and timing of krill maturation in the wild.

- Krill have evolved control mechanisms to perceive seasonal variations in light conditions, and thereby, to anticipate the succession of the season.
MELATONIN AND THE TRANSDUCTION OF PHOTOPERIODIC INFORMATION

The indoleamine melatonin was hypothesized to be involved in the transduction of photoperiodic information in krill. A seasonally changing level of melatonin secretion is thought to mediate the effect of photoperiodic changes for the organization of annual or circannual rhythms in krill physiology. To address this issue, the occurrence of melatonin in krill and its possible role in mediating seasonal metabolic changes is evaluated on the basis of field samples (manuscript III).

The findings indicate that krill exhibit an annual rhythm of metabolic activity with high rates in summer, and low or reduced rates in winter, but also experience no mediating role of melatonin in the control of seasonal metabolic changes. Overall, the results suggest that krill generally lack melatonin secretion on a diurnal, as well as on a seasonal, basis which then may be used to time daily and/or seasonal functions. These findings have important implications for further understanding of the mechanism by which the Southern Ocean light regime affects the physiology of krill. It can be concluded that the proposed photoperiodic response system in krill is not based on melatonin as a transducer of photoperiodic information, although melatonin is assumed to be principally involved in the transduction of photoperiodic information in living organisms (Vivien-Roels and Pévet 1986), and has been detected in the visual system of several crustacean species (Tilden et al. 1997; Tilden et al. 2001; Agapito et al. 1995; Balzer et al. 1997). For the organisation of annual rhythms in krill physiology, however, timely prediction of the seasonal period is crucial. There is now some evidence that organisms use the tools of the circadian system to sense changes in daylength and to mediate the photoperiodic response (Oster et al. 2002; Schultz et al. 2003).

Principally, three main models for photoperiodic time measurement in organisms can propose mechanisms for the generation of annual rhythms of physiological functions in krill, with two of them being based on the involvement of a circadian system. The first model assumes the gradual accumulation of a chemical product in the organism (“hourglass” model). A certain quantity of this product is necessary to trigger a physiological response. The chemical product may be degraded in darkness and accumulates during the day, or it may be degraded by light and accumulates during the night. If the critical night length (or day length) is achieved, the threshold is reached and a physiological response is initiated. This model, however, lacks an endogenous rhythmicity of the chemical product and thus of the involvement of a circadian system (Lees 1973). The other two models assume a role of a circadian system, and are further classified into the external and internal coincidence model.
In the first, light has a dual role: it entrains a circadian rhythm (e.g. production of a photosensitive product) and also acts as a photoperiodic stimulus (Bünning 1936; Davis 2002). Because of the seasonal variation in photoperiod, the circadian rhythm becomes periodically exposed to the external stimulus (light) which then triggers a physiological response. Thus, photoperiodic reaction depends on the coincidence between light and a particular phase of the circadian rhythm. In the second model, light’s only role is the entrainment of multiple circadian rhythms (Pittendrigh et al. 1984; Davis 2002). Changes in photoperiod thus cause a different phase relationship between two or more circadian rhythms within the organism. Without a direct inductive effect of photoperiod, physiological reaction will be triggered when a particular phase relationship is established, for example, the circadian rhythm of an enzyme coincides with the rhythm of its cofactor. These general models of photoperiodic time measurement can propose mechanisms both for the generation of annual rhythms of physiological functions in krill and the synchronization of endogenous circannual rhythms of krill physiology to the external annual cycle of photoperiod.

Conclusions of manuscripts III:

- Krill exhibit an annual rhythm of metabolic activity with high rates in summer and low or reduced rates in winter.

- There is no mediating role of melatonin in the control of seasonal metabolic changes.

- Krill generally lack melatonin secretion on a diurnal as well as on a seasonal basis, which then may used to time daily and/or seasonal functions.

- The photoperiodic response system in krill is not based on melatonin as a transducer of photoperiodic information.
PERSPECTIVES

The main focus of this thesis is to gain first insights into the effects of seasonal light conditions on the physiology of krill. During the course of this study several new questions emerge which remain unresolved. Further experimental studies on krill, devoted to these questions, are required to allow us to build up a more detailed picture of the mechanisms by which the natural light regime affects the physiology of this key species in the Southern Ocean.

A critical topic is the specific effect of light conditions on physiological functions in krill. In our study, the effect of simulated light regimes on certain life-cycle parameters of krill has been shown. However, this study focused on a simulation of constant Antarctic summer, autumn, and winter light regimes over a period of three months. Moreover, the light condition in the different simulated seasons differed in light duration and intensity. Therefore, it remains unclear whether the observed effects are the result of differences in photoperiod or light intensity. The annual cycle in photoperiod is the major source of predictive environmental information in the control of a variety of seasonal activities in numerous animals. It is very important to know whether, and to what extent, life-cycle parameters of krill are synchronized to the seasonal cycle of photoperiod. Thus, it is now essential to expose krill to a simulation of an annual course of Southern Ocean photoperiod and investigate the correlation between seasonal changes in light duration and seasonal changes in the development of krill.

In this context, it is crucial to evaluate the response mechanism to differences in light conditions. The present results indicate that the progressive development of krill maturity most likely represents a circannual rhythm which is synchronized with the natural year by the annual course of light conditions. However, this conclusion can only be drawn on the basis of previous studies, and it remains unclear at this stage if the annual course of photoperiod is used as a synchronizer to link the inherent rhythm to the natural year. Moreover, it is unclear if seasonal changes of feeding and metabolic activity of krill also represent a circannual rhythm, or may be controlled directly by the annual course of photoperiod. To investigate the contribution of a circannual clock to the control of circannual rhythms in life-cycle parameters of krill, we need to establish under free running conditions, whether krill show endogenous rhythms or not. Laboratory simulations of a shortened (6 months) annual course of photoperiod will then help us to investigate if the annual course of photoperiod is used as a synchronizer to link the inherent rhythm to the natural year.
The results of this thesis clearly show that the proposed photoperiodic response system in krill is not based on melatonin as a transducer of photoperiodic information, and open the question how photoperiodic time measurement is generally realized in krill. There is now some evidence that organisms use the tools of the circadian system to sense changes in daylength and to mediate the photoperiodic response. Consequently, a central question is whether there is evidence for a circadian rhythmicity in krill which may be involved in the mechanism of photoperiodic time measurement, and thereby in the process of generation of annual rhythms, or synchronization of circannual rhythms with the natural year. Circadian clocks have evolved in many organisms to adapt to the oscillation of the day-night cycle and to predict daily events for the synchronization of physiology and behaviour to the environment. Thus, the presence of a circadian clock in krill is extremely likely but has not been proven yet. Recent findings about the molecular mechanisms of circadian clocks reveal that they are generated by the rhythmic expression of genes encoding for proteins that represent the molecular gears of the clock. Therefore, a future approach must be to investigate the molecular mechanisms for generation of a circadian and/or circannual rhythm in krill. The identification of rhythmic genes, and their associated products that are involved in daily and/or seasonal rhythms exhibited by krill, is essential to understand the mechanisms of synchronization between the seasonal development of krill and the seasonal cycles of environmental features in the Southern Ocean.

**Perspectives**

- To study the effect of photoperiod on the seasonal development of Antarctic krill:
  - How are different life-cycle parameters of krill synchronized to the annual course of Southern Ocean photoperiod?
  - What is the contribution of a circannual clock to the control of circannual rhythms in life-cycle parameters of krill?
  - Is the annual course of photoperiod used as a synchronizer to link an endogenous rhythm to the natural year?

- To investigate the molecular mechanisms for generation of a circadian and/or circannual rhythm in krill:
  - Which genes and proteins are involved in daily and/or seasonal rhythms exhibited by krill?
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SUMMARY

Antarctic krill (*Euphausia superba*) is the most abundant of the world’s euphausiids and plays a central role in the Southern Ocean pelagic ecosystem. The unique position of krill in the food web, the increasing commercial interest on this species, and its susceptibility to global climate change emphasize the urgency of understanding krill life history and biology. With respect to the complex life cycle of krill that has evolved to exploit a highly seasonal environment, it is crucial to understand krill adaptations to its environment and the forces that determine it. In the course of the year, important life-cycle parameters of krill such as metabolic activity and maturity constitute seasonal (annual) patterns that specify the time for reduced metabolic and reproductive activity. Reduction of metabolic and feeding activity of krill during the Antarctic winter is suspected to be an overwintering strategy to survive the times when food is scarce. The onset and timing of krill maturation is important for the coincidence of the reproductive period with times of substantial food supply, and guarantees the reproductive success of krill. However, current uncertainties exist regarding the mechanisms causing synchronization between the seasonal development of krill and the seasonal cycles of environmental features. The main focus of this thesis was to evaluate the influence of seasonal light conditions on the physiology of krill, particularly with regard to the over-winter biology and maturity development of krill.

The effect of different simulated Southern Ocean light regimes on feeding and metabolic activity, as well as on the development of sexual maturity, of krill was evaluated on the basis of laboratory experiments on live krill. It could be demonstrated that different experimental light regimes, simulating Southern Ocean summer autumn/spring (hereafter autumn) and winter light conditions, had an important effect on the physiology of krill. Krill exposed to summer and autumn light conditions showed an increase in feeding activity, oxygen consumption, and activity of the metabolic enzyme malate dehydrogenase (MDH) over the experimental period. Parameters of krill held under autumn light condition showed a more consistent increase and remained below those of krill held under summer light condition. No change was recorded for krill exposed to winter light condition; feeding activity did not respond to high food availability, and oxygen consumption rates and MDH activity were significantly (*p* < 0.05) lower than those of krill exposed to summer light condition. The results indicate that, irrespective of food supply, the level of feeding and metabolic activity in krill correlates with the degree of prevailing light conditions. Thus, a summer light regime of prolonged photoperiod and enhanced light intensity implicates higher feeding and metabolic
activity than a more attenuated light regime of autumn condition, or even more, of a winter light regime. In this way, there is evidence that reduced values of feeding and metabolic activity of krill during winter are not caused directly by the scarcity of food at this time of the year, simply reflecting a change in ingestion rate, but instead represent an inherent adaptational overwinter strategy triggered by the Southern Ocean light regime.

The sexual maturity of krill exposed to summer and autumn light conditions showed an accelerated succession of external maturity stages during the experimental period. Krill exposed to autumn light condition showed a more subtle development of maturity, while krill exposed to winter light conditions showed no changes in external maturity during the course of the study. The results indicate that the succession of female and male krill maturity stages is accelerated by light conditions of prolonged photoperiod and enhanced light intensity. This shows that the development of maturity does not simply reflect energy availability, and is not directly keyed to high food concentrations in the water column, typically occurring during the summer phytoplankton blooms. In this way, there is evidence that light conditions in the Southern Ocean play an important role to adjust the onset and timing of krill maturation in the wild and thus in the adaptation of krill reproduction to a highly seasonal environment.

Together, the results indicate that seasonal (annual) rhythms in life-cycle parameters of krill do not generally reflect the annual cycle of food concentration in the environment. Instead, the results indicate that, beside krill’s potential for physiological plasticity, these rhythms are more the result of fundamental seasonal adaptations in the animal physiology. This emphasizes the role of the Southern Ocean light regime as an essential cue governing the seasonal cycle of krill physiology, and gives evidence that krill have evolved control mechanisms to perceive seasonal variations in light conditions and thereby to anticipate the succession of the season. This enables krill to regulate its physiology due to the pronounced seasonal differences in environmental conditions.

The indoleamine melatonin was hypothesized to be involved in the transduction of photoperiodic information in krill. To address this issue, the occurrence of melatonin in krill and its possible role in mediating seasonal metabolic changes was evaluated on the basis of field samples during the Antarctic winter and summer. The results show seasonal variations in the metabolic status of krill and demonstrate that krill was in a state of reduced metabolic activity during winter. It is further shown that, neither during winter nor during summer, were there detectable melatonin concentrations in krill. The findings confirm that krill exhibit an annual rhythm of metabolic activity with high rates in summer and low or reduced rates in winter but also show no mediating role of melatonin in the control of seasonal metabolic
changes. These findings have important implications for further understanding of the mechanism by which the Southern Ocean light regime affects the physiology of krill. It can be concluded that the proposed photoperiodic response system in krill is not based on melatonin as a transducer of photoperiodic information, although melatonin is assumed to be principally involved in the transduction of photoperiodic information in living organisms and has been detected in the visual system of several crustacean species.
ZUSAMMENFASSUNG


Schluss zu, dass Krill Kontrollmechanismen entwickelt haben muss, die es ihm ermöglichen
die saisonale Variation der Lichtbedingungen zu antizipieren und somit den zeitlichen Verlauf
der Saison zu bestimmen. Dies wiederum versetzt Krill in die Lage seine Physiologie an die
ausgeprägten saisonalen Unterschiede der Umweltbedingungen anzupassen.

Das Indolamin Melatonin wurde in diesem Zusammenhang mit der Übersetzung der
photoperiodischen Information in Verbindung gebracht. Auf der Grundlage von Krillproben
aus dem antarktischen Winter und Sommer wurde das Vorhandensein von Melatonin und
seine mögliche Rolle in der Vermittlung saisonaler metabolischer Veränderungen überprüft.
Die Messungen zeigten saisonale Unterschiede im metabolischen Status der Tiere, wobei
Krill aus dem Winter einen reduzierten Stoffwechsel aufzeigte, jedoch konnten weder im
Sommer noch im Winter nachweisbare Melatoninkonzentrationen im Krill gefunden werden.
Diese Ergebnisse bestätigen zum einen den jährlichen Rhythmus der metabolischen Aktivität
von Krill, der zwischen hohen Werten im Sommer und niedrigen Werten im Winter wechselt,
zeigen aber auch zugleich das Melatonin bei der Kontrolle dieser saisonalen Veränderungen
keine Rolle spielt. Diese Ergebnisse haben wichtige Auswirkungen auf das Verständnis der
Mechanismen durch die das Lichtregime im südlichen Ozean die Physiologie von Krill
beeinflusst. Obwohl angenommen wird das Melatonin in allen lebenden Organismen eine
wichtige Rolle bei der Übersetzung von photoperiodischen Signalen spielt und bereits in
zahlreichen Crustaceen Arten nachgewiesen wurde, kann hiermit eine Beteiligung von
Melatonin bei der photoperiodischen Signalverarbeitung in Krill ausgeschlossen werden.
DANKSAGUNG

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ERKLÄRUNG

Gem. §6 (5) Nr.1-3 Promotionsordnung

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