# Overwintering of the hoverfly *Episyrphus balteatus*: Long-distance migration or local overwintering?

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# Abstract

The objective of this study was to obtain a better understanding of two important sections of the life history of a widespread aphidophagous hoverfly with supposed migratory behaviour, *Episyrphus balteatus* (DeGeer) (Diptera: Syrphidae). Therefore, the study focuses on the overwintering in northern Germany and on long-distance migration in Europe, both of major importance in terms of efficacy of this predator in aphid control (e.g., predator-prey synchronisation in spring).

In the first part, a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis using mitochondrial (A+T-rich region; mtDNA) and genomic (*zen*-region; nDNA) DNA was performed on 182 female individuals of *E. balteatus*. Specimens originated from 13 sampling sites in six European countries. The analyses revealed 12 and 18 haplotypes, respectively, for the two DNA types, several of them with a wide distribution, although seven and eight haplotypes, respectively, occurred only in one location. In contrast to other studies on mobile insects, the genetic diversity was relatively high. However, lack of population subdivision, low genetic distances between populations, the very high gene flow rates, and the complete lack of isolation by distance suggest that *E. balteatus* populations are largely connected and that there is an absence of large-scale geographic structuring. These results support the hypothesis that *E. balteatus* is a migratory hoverfly species, capable of moving over large distances.

In the second part, the overwintering biology of *E. balteatus* was studied and analysed. In Europe, females of this species can overwinter as adults in a facultative, reproductive diapause. The diapause stage is characterized by the ovaries ceasing to develop and by hypertrophy of the fat body. Diapause was induced during the second and third larval instars. The critical photoperiod for inducing diapause was approximately 11.9h, corresponding to the day lengths that occur during end of September in Hannover, Germany. When temperatures were lower, insects could be induced into diapause at longer day lengths, similar to those occuring in mid-September in Hannover. A semi field study was done during one winter to confirm the results obtained under laboratory conditions and to obtain additional information on the overwintering development and mortality of *E. balteatus*. The results suggest that overwintering mortality was correlated with the duration of the experiment and with humidity, rainfall, and temperature.

In a further approach, the effects of various low temperatures on survival times of different treated males and females (diapausing, acclimated, both diapausing and acclimated, untreated) were investigated (lethal time analysis). The laboratory experiments imply low cold-hardiness in *E. balteatus*. Diapausing and acclimated females were the most cold hardy stage and females were generally more cold hardy than males. However, in particular the diapause stage serves to avoid energy exhaustion at higher temperatures (up to 0°C) and acclimatisation to prevent chilling injuries at sub-zero temperatures above the supercooling point. With increasing duration of low temperatures and declining temperature, mortality was strongly increasing in all experiments. As mortality factors during low temperatures, chilling injuries, desiccation injuries and energy exhaustion were most likely. Additionally, this species did not synthesise and accumulate any cryo-protectants. Consequently, overwintering sites seem to play an important role as buffered shelter against too low temperatures, strong temperature fluctuations, and inoculative freezing, but have still to be detected.

To conclude, *E. balteatus* is poorly adapted to local overwintering in central and northern Europe and this strategy involves a very high risk of mortality. As a result, this species may have a status between a chill susceptible and chill tolerant insect. Thus, a double strategy of local overwintering and southward large-scale migration is likely as it would spread the risks and possibly increases the chances of survival. The importance of these results for cereal aphid control is discussed.

**Keywords**: Diptera, Syrphidae, population genetics, migration, reproductive diapause, overwintering, cold hardiness, conservation biological control

# Zusammenfassung

Ziel dieser Arbeit war es, ein besseres Verständnis von zwei wichtigen Abschnitten im Lebenszyklus von *Episyrphus balteatus* (Diptera: Syrphidae) (DeGeer), einer weitverbreiteten aphidophagen Schwebfliege, von der man annimmt das sie migriert, zu gewinnen. Die Arbeit setzt daher einen Schwerpunkt auf die lokale Überwinterung in Norddeutschland und auf Fernmigrationen im europäischen Raum, beides Faktoren, die die Effizienz dieses Prädators bei der Kontrolle von Blattläusen maßgeblich beeinflussen (z. B. über die Synchronisation zwischen Räuber und Beute im Frühjahr).

Im ersten Teil wurde die PCR-RFLP Reaktion unter Verwendung von zwei polymorphen DNS-Regionen (mitochondriale DNS: A+T-rich region; nukleare DNS: *zen*-region) an 182 Weibchen von *E. balteatus* verwendet, die von 13 Herkünften aus sechs europäischen Ländern stammten. Bei den Analysen wurden 12 beziehungsweise 18 Haplotypen für die beiden DNS-Typen gefunden, von denen mehrere eine weite Verbreitung hatten, während 7 bzw. 8 Haplotypen nur einmal vorkamen.

Im Gegensatz zu anderen Studien über mobile Insekten, war die genetische Diversität von *E. balteatus* relativ hoch. Jedoch deuten das Fehlen von unterscheidbaren Subpopulation, die niedrigen genetischen Distanzen zwischen Populationen, die sehr hohen Genfluss-Raten und das Fehlen von ‚Isolation by Distance' darauf hin, das Populationen von *E. balteatus* im hohen Maße vernetzt sind und großräumige geographische Populationsstrukturen fehlen. Außerdem wird die Hypothese gestützt, dass *E. balteatus* eine migrierende Schwebfliege ist, die große Entfernungen überwinden kann.

Im zweiten Teil wurde die Überwinterungsbiologie von *E. balteatus* untersucht. In Europa können nur Weibchen als adultes Tier in einer fakultativen, reproduktiven Diapause überwintern. Das Diapause-Stadium ist durch die fehlende Ovarienentwicklung und die Hypertrophie des Fettkörpers charakterisiert. Die Induktion der Diapause erfolgt ausschließlich im zweiten und dritten Larvenstadium. Als kritische Photoperiode für die Induktion wurden ca. 11,9h ermittelt, eine Tageslänge die im Raum Hannover etwa Ende September erreicht wird. Bei niedrigen Temperaturen verschob sich die Diapause-induktion zu längeren Tagen, wie sie schon Mitte September erreicht werden. Während eines Winters wurde ein Semi-Freiland Experiment durchgeführt, um die Laborergebnisse zu überprüfen und um zusätzliche Informationen bezüglich Überwinterungsmortalität und Phänologie zu erhalten. Die Ergebnisse zeigten, dass die Mortalität während der

Überwinterung mit der Dauer des Experimentes sowie Luftfeuchtigkeit, Niederschlag und Temperatur korreliert war.

In einem weiteren Ansatz wurden die Auswirkungen von verschiedenen niedrigen Temperaturen auf die Überlebenszeiten von verschieden vorbereiteten männlichen und weiblichen *E. balteatus* untersucht (sogenannte ‚lethal time'-Analysen). Diese Laborexperimente deuten darauf hin, dass *E. balteatus* lediglich eine niedrige Kältehärte besitzt. Akklimatisierte Weibchen in Diapause waren die kältehärtesten Tiere, außerdem waren Weibchen generell kältehärter als Männchen. Das Diapause-Stadium diente insbesondere dazu, den vorzeitigen Verbrauch der Energiereserven bei höheren Temperaturen (bis 0°C) zu vermeiden und Akklimatisierung diente dazu ‚chilling injuries' unter 0°C aber über dem Unterkühlungspunkt dieser Tiere zu vermeiden. Je länger die Versuchstiere niedrigen Temperaturen ausgesetzt waren und niedrigere Temperaturen generell führten zu stark ansteigender Mortalität in allen Versuchen. Mögliche Faktoren die zur Mortalität bei niedrigen Temperaturen beitrugen, waren insbesondere ‚chilling injuries', Schäden durch Austrocknung und Energiemangel. Darüber hinaus synthetisiert bzw. akkumuliert *E. balteatus* keinerlei Gefrierschutzsubstanzen. Folglich hat diese Art wahrscheinlich einen Status zwischen einem kälteempfindlichen (‚chill susceptible') und einem kältetoleranten ('chill tolerant') Insekt. Überwinterungsverstecke scheinen daher eine bedeutende Rolle als Schutz gegen zu niedrige Temperaturen, zu hohe Temperaturfluktuationen und auch ‚inoculative freezing' (d. h. Gefrieren durch äußere Eiskeime) zu spielen, obwohl sie immer noch nicht gefunden worden sind.

Daraus folgt abschließend, das *E. balteatus* nur schlecht an die lokale Überwinterung in Mittel- und Nordeuropa angepasst ist und diese Strategie ein sehr hohes Mortalitätsrisiko beinhaltet. Aus diesem Grund ist eine Doppelstrategie aus lokaler Überwinterung und südwärts gerichteter Fernmigration wahrscheinlich, da dieses die Risiken verteilen und so die Überlebenschancen erhöhen würde. Die Bedeutung der Ergebnisse für die Regulierung von Getreideblattlauspopulationen wird diskutiert.

**Schlagworte**: Diptera, Syrphidae, Populationgenetik, Migration, reproduktive Diapause, Überwinterung, Kältehärte, Nützlingsförderung

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# 1. General Introduction

High-input agriculture shares the same characteristics in developed countries all over the world; examples are monocultures of high-yield cultivars, extensive use of fertilizers and pesticides, large-scale farms, rapid technological innovation and the enlargement of plots appropriate for mechanized food production. The latter resulted in fragmented landscapes, which have often replaced patchwork landscapes in which many small annually cropped plots are embedded in a matrix of semi-natural habitats with higher ecological value. This reshaping is a result of so-called consolidation of arable land by means of the removal of hedges, woodlots, grassland stripes, and other perennial habitats and crop edges (e.g., Jedicke, 1994; Dover 1997; Kühne & Freier, 2001; Robinson & Sutherland, 2002; Clay, 2004).

Apart from the favourable effects of these measures, the tremendous gains in productivity and efficiency (e.g., FAO, 2001) which was politically desirable for many decades, since several years the drawbacks of this type of agriculture have emerged as major issue and public is becoming aware of these concerns. Examples of these drawbacks are the loss of biodiversity (in plant and animal species (species diversity) and habitats (ecosystem diversity)) and plant protection problems such as pest outbreaks or developing resistance of pests and diseases against plant protectants (Matson et al., 1997; Krebs et al., 1999; Benton et al., 2002; Tilman et al., 2001, 2002).

As a consequence, various alternative agriculture systems and a wide array of measures developed that try to avoid the problems of intensive farming systems. These systems are termed ‘sustainable’ and ‘low-input farming’ systems as for example

'biodynamic agriculture' or 'organic farming'. Organic farming for example is a holistic production management system that promotes and enhances agro-ecosystem health, including functional diversity, biological cycles, and soil biological activity in preference to the use of external inputs (IFOAM, 2007). With respect to plant protection, this is accomplished by using, where possible, agronomic, biological, and mechanical methods (e.g., planting & harvesting date manipulation, clean cultivation, intercropping, and biological control agents such as natural enemies), as opposed to using synthetic materials (e.g., pesticides, fertilizers).

Although today the benefits of these types of agriculture are accepted and since a reform in 2003/2004 they are part of the Common Agricultural Policy (CAP) of the EU, socio-economics still promote and subsidise in the first place high-input agricultural systems (e.g., Prazan, 2002). As a result, in the EU most arable land is still cultivated using intensive agriculture measures and less than five percent of the agricultural area is used for certified organic farming systems. Nevertheless, its importance is growing in the last years strongly (Commision Européenne, 2005) and most EU countries grant organic farming with various EU and national subsidies and agri-environment measures (EU, 2005).

Various studies suggested that increasing biodiversity generally amounts to higher stability and resilience of ecosystems (see Andow, 1991; Hooper et al., 2005). Agro-ecosystems on the other hand, whose natural regulation capabilities are reduced and largely replaced by human regulation mechanisms, are estimated as instable compared to natural or semi-natural ecosystems making them more vulnerable for example to pest outbreaks (Weigmann, 1987; Cardinale et al., 2003; Tscharntke et al., 2005). Moreover, the effect of increased antagonist diversity on herbivore population suppression is

nowadays confirmed (Elliott et al., 1999, Landis et al., 2000; 2002a,b; Kean et al., 2003; Snyder et al., 2006).

This is the starting point of another approach in plant protection, the promotion and conservation of natural enemies already existing in the agro-ecosystem. This so-called 'conservation biological control' includes all procedures that modify environmental conditions in order to reduce or eliminate conditions unfavourable to natural enemies or to provide resources that promote population growth, recruitment and performance of them (Ruberson et al., 1999). Especially plot realignments have been shown to have a negative impact on a variety of natural enemies by reducing the amount of perennial habitats and crop edges (e.g., Kühne & Freier, 2001; Ries et al., 2004; Pfiffner et al., 2005). But also side-effects of pesticides, the regular disturbance by tillage, and the devastation of patches have detrimental effects on the numbers of natural enemies, simply by killing beneficial insects, by removing food resources for adults (e.g., flowers), and killing alternative food resources (e.g., aphids on weeds) for predatory stages. Hence, it is the approach of conservation biological control to support local natural enemies in this dynamic and hostile environment and to give them a better opportunity to regulate pests.

For this it is obligatory to improve not only conventional control strategies (e.g., by more selective pesticides, reduced amounts and reduced drift of pesticides) but also to re-establish a more heterogeneous landscape leading to increased biodiversity (i.e., habitat management). Permanent habitats comprise several functions for natural enemies, they serve for example as shelter, overwintering site, alternative food resource, or starting point for reinvasion into the annually cropped fields (Dempster & Coaker, 1974; Corbett & Rosenheim, 1996; Salveter, 1996; Tylianakis et al., 2004; Landis et al., 2005).

The life histories of insect species successful colonizing and reproducing in agricultural landscapes need to be adapted to the high spatio-temporal variability of

habitats and the frequent disturbances. Often these species need to complete their life cycle while conditions are favourable (Southwood, 1988). Therefore, the typical inhabitants of these highly disturbed and ephemeral habitats are influenced by r-selection, characterized by large numbers of offspring (i.e., high fecundity) with high immature mortality, fast development, small size, and high dispersal rates (e.g., Stearns, 1976; Price 1984). Due to the high dispersal power and frequency, these species can exploit the temporary resources in ephemeral habitats and also persist even though their habitats are frequently destroyed (e.g., Fahrig & Merriam, 1994). This includes the hoverfly species *Episyrphus balteatus* (DeGeer, 1776), a common and very mobile hoverfly, capable of colonizing many different habitats and climatic zones. It can be found from Palearctic, Afrotropical, and Oriental to Australian regions and is only absent in the Americas (Peck, 1988; Torp, 1994). This species can colonize and reproduce successful in fragmented landscapes and easily disperse between local habitats (e.g., aphid infested plants, blooming flowers and trees, refuges (shade), or hovering sites for mate seeking) (e.g., Salveter & Nentwig, 1993; Sutherland et al., 2001; Wratten et al., 2003). The success is attributed to its high ecological valence (e.g., polyphagous larvae, euryanthy, eurytopic demands in terms of climate and habitat) and behavioural characteristics such as sensitive search mechanisms for prey and numeric and functional responses to increasing prey densities (e.g., Tenhumberg, 1995; Bargen et al., 1998). Therefore, *E. balteatus* is regarded as one of the most important antagonists in the control of cereal aphids in some parts of Europe (e.g., Ankersmit et al., 1986; Chambers & Adams, 1986; Schier, 1988; Tenhumberg, 1992; Salveter, 1996; Krause, 1997).

Successful conservation biological control measures and the promotion of beneficial species require of course detailed knowledge of the biology and ecology of the antagonists, the pests, and their interactions (e.g., Lewis et al., 1997; Verkerk et al., 1998).

Though until now many studies on this subject were carried out, results are often ambiguous and it is difficult to generalize them. This is caused by the complexity of the subject, as there are numerous influencing factors as for example weather conditions, herbivore and antagonist ratios, effects of scale, year-to-year variation in population densities, or interactions between beneficials (Andow, 1991; Lucas et al., 1998; Thies & Tscharntke, 1999; Norris & Kogan, 2005).

Thus, despite the high efforts in research, only a minor part of the relevant species is well known and also in these species there are several knowledge gaps. Out of these studies it remains often unclear if heterogeneous landscapes really increase the exchange between plot and adjacent habitats, which population densities of beneficials are existent and sufficient for control, which distances beneficial insects can move, to what extent other factors (e.g., fecundity, host specificity, or searching capacity of beneficials) have an impact on populations, which microclimatic preferences natural enemies have, or where they overwinter (e.g., Lewis et al., 1997; Bugg & Pickett, 1998; Schmidt et al., 2003).

The hoverfly *E. balteatus* is a good example how complex these relations can be. Although *E. balteatus* can be considered as well known, regarding plant protection aspects, the reasons for regional differences in success or failure of aphid control by this species are not very well understood. For example in the western parts of Germany and in the Netherlands, it is the most important natural enemy of cereal aphids, whereas in the eastern plains of Germany (Magdeburger Börde) other taxa such as ladybirds seem to be more important (Büchs, 2001).

Another problem is that in northern parts of Germany cereal aphid control often fails, whereas in the southern parts it is successful (e.g., Poehling et al., 1991). Syrphids generally are the first that can attack aphid colonies in spring, due to their life history with low developmental temperature thresholds and adult overwintering (Dixon et al., 2005). It was found that for the success of cereal aphid control the synchronization in time and

space between prey (aphids) and predator (syrphid larvae) is crucial. In studies over several years, a good relation between maximum aphid densities and the number of aphids during the first occurrence of syrphid larvae was found (Tenhumberg & Poehling, 1995). Early oviposition of syrphids regularly leads to successful cereal aphid control, indicating the importance of good synchronization in time. Nevertheless, the first occurrence of especially adults of *E. balteatus* changed significantly between years and regions. In particular, in southern parts of Germany and in regions with more heterogeneous landscapes, densities were higher compared to northern Germany and regions with lower landscape diversity (Poehling et al., 1991; Tenhumberg, & Poehling, 1995; Krause & Poehling, 1996). For this phenomenon, two theories were developed to explain this regular occurring regional pattern.

First *E. balteatus* is considered as a migratory species, shifting regularly between the Mediterranean region as overwintering and central to northern Europe as main breeding site. This hypothesis is based on quite a lot of occasionally observations for example in mountains, coasts, and on ships (see Gatter & Schmid, 1990, and references therein), and two long-term studies where large numbers of southward moving individuals were caught in late summer and autumn by interception traps (Aubert et al., 1976; Gatter & Schmid, 1990).

The second hypothesis involves local overwintering of adults in Central Europe. It is derived from flight observations from late autumn to spring and from early oviposition near aphid colonies on trap plants and aphid-infested plants in hedges and gardens in early spring (e.g., Schier, 1988; Krause & Poehling, 1996; Salveter, 1996). Both can only be explained by local hibernation of residents, because immigrating hoverflies are expected in June (van der Goot, 1979).

Accordingly, several components of the life history of *E. balteatus* that are important for understanding the biology, the prediction of control success, and the

enhancement of this species aare still unclear. The objectives of this study were therefore twofold:

The first aim was to elucidate population structure and gene flow from samples of various European origins with polymorphic genetic markers (PCR-RFLPs) using different data analysis techniques as for example genetic distances, population structure (F-statistics), AMOVA, genetic exchange among populations (Nm), and isolation by distance.

And secondly, to investigate if this species is in general able to overwinter locally in northern Germany and to detect its physiological adaptations for overwintering as diapause, cold hardiness, and cryo-protectant production using laboratory and semi-field experiments.

# 2. Restriction fragment-length polymorphisms of different DNA regions as genetic markers in *Episyrphus balteatus*

## 2.1 Introduction

*Episyrphus balteatus* (DeGeer) is considered in Europe to be one of the most important natural enemies of economically important aphids, because of its periodically high abundance, the voracious feeding habits of the larvae, the efficient prey finding abilities and high mobility of the adults (e.g., Aubert et al., 1976; Ankersmit et al., 1986; Tenhumberg & Poehling, 1995; Bargen et al., 1998). Despite this importance for biological control, little is known on the population genetics and overwintering biology of *E. balteatus* with and regard to the latter, two hypotheses exist which have been the subject of substantial debate in the literature (e.g., Krause & Poehling, 1996; Salveter, 1996; Hart & Bale, 1997b). First, *E. balteatus* is considered to be a migratory species, regularly moving between overwintering sites in the Mediterranean to central and northern Europe as main breeding sites. This hypothesis is mainly based on occasional flight observations, e.g., on mountains, along coasts, and on ships (see Gatter & Schmid, 1990, and references therein), and two long-term studies where large numbers of southward moving individuals were caught in late summer and autumn by interception traps in the Alps (Aubert et al., 1976) and south-western Germany (Gatter & Schmid, 1990). The second hypothesis postulates local overwintering of adult *E. balteatus* in Central Europe. It is derived from flight observations e.g., in northern Germany (Krause & Poehling, 1996) or Switzerland (Salveter, 1996) in late autumn to early spring, which were explained as indicating local hibernation by non-migrating individuals. Such residents go through a facultative diapause in which they attain some cold resistance (see chapter 3.6.2) as an adaptation to hibernation (Tauber et al., 1986).

Possibly both migrants and locally hibernating individuals contribute to the establishment of the *E. balteatus* summer populations in Central Europe. However, even under such an assumption, various questions remain open: are populations or sub-populations connected? To what extent are populations subdivided and distinguishable? What is the genetic variability and diversity of populations and/or sub-populations? What are the sources for the high abundance of *E. balteatus* during the summer months in Central Europe? What distances can migrants travel?

Hence the purpose of this study: to investigate if, and to what extent, European populations of *E. balteatus* are connected (in this paper individuals from one sample site are referred to as a population, even though these samples may be part of one larger population). As direct methods such as mark and recapture techniques or migration observations turned out not to be useful or feasible for hoverflies (Salveter & Nentwig, 1993; Nathan et al., 2003) indirect methods such as molecular markers were chosen, since they are reliable and can have a sufficiently high resolution, allowing detection of even small genetic differences within and between populations (Avise, 1994; Loxdale & Lushai, 1998).

The genetic structure of populations is determined by the balance between genetic drift, mutation, natural selection and gene flow (Roderick, 1996). Gene flow tends to homogenize the genetic structure of populations (e.g., Bohonak, 1999; Mun et al., 1999), although the effects of two-way migration are still little known (Freeland et al., 2003). Nevertheless, gene flow can also be restricted in highly mobile species like *E. balteatus*, e.g., by physical barriers (mountains, seas), isolation by distance or habitat patchiness (e.g., Loxdale & Lushai, 2001). In addition, founder effects may also influence population structure (Hedrick, 2000).

In this paper the genetic characterisation of a migratory hoverfly species collected over a broad geographical range is presented for the first time. The study involved a

restriction fragment length polymorphism analysis (PCR-RFLP) of two DNA regions: the A+T-rich region (control region), a non-coding region of mitochondrial DNA (mtDNA) near the origin of replication of the molecule. Because of the comparatively rapid evolution and lack of recombination, mtDNA has been used extensively in population genetic studies (Avise, 1994; Loxdale & Lushai, 1998). The A+T-rich region is one of the most variable parts of mtDNA (Simon et al., 1994; Ballard & Whitlock, 2004) and has been useful for resolving several population genetic questions (e.g., Brower & Boyce, 1991; Parker et al., 1998; Schultheis et al., 2002). To overcome certain disadvantages of mtDNA, e.g., only maternal gene flow, only one non-recombinant locus, and lower effective population size, another DNA region was also selected, namely a nuclear DNA region representing a derivative of the Hox genes. Hox genes are a set of related genes encoding homeodomain transcription factors, necessary for developmental regulation (Hughes & Kaufman, 2002). In this study a part of a Hox3 gene homolog called "*zerknüllt*" (*zen*), required for establishment of extraembryonic tissue (Stauber et al., 2002), was used. Using these methodological approaches, it was intended to identify patterns of population differentiation and to estimate genetic variability between European populations of *E. balteatus*, to analyse the genetic diversity within and among populations, to reveal the degree of connection of populations by gene flow, and to study the origin of the high numbers of individuals frequently observed in the summer months in Central Europe.

## 2.2 Material and methods

### 2.2.1 Samples

Adult female flies only were used in this study since they are primarily the ones that migrate and overwinter (Aubert et al., 1976; Gatter & Schmid, 1990). Samples from several European countries were collected by hand-net and immediately transferred to ethanol (70-98%). Samples from the island of Helgoland and Berlin (both Germany) were from Malaise traps (trapping fluid ethylene glycol) and transferred in ethanol (70-98%) after emptying the trap. After shipping, all material was stored in absolute ethanol and frozen at -20°C until further use. More detailed information on locations and collection dates are summarised in table 1.

**Table 1:** Locality, individual numbers and dates of all *Episyrphus balteatus* samples.

| Locality (Abbreviation) | Longitude | Latitude | Individual Number | Date of Sampling |
|---|---|---|---|---|
| Germany | | | | |
| Helgoland (Hel) | 07°50´E | 54°10´N | 10 | VII.02* |
| Harz-Braunlage (Hrz1) | 10°40´E | 51°40´N | 21 | 27.VII.02 |
| Harz-Kamschlacken (Hrz2) | 10°30´E | 51°50´N | 15 | 27.VII.02 |
| Schleswig-Holstein (S-H) | 10°20´E | 53°40´N | 7 | 05.VIII.02 |
| Elm (Elm) | 10°50´E | 52°10´N | 18 | 28.VII.02 |
| Münster (Mue) | 06°40´E | 51°50´N | 8 | 15.VII.02 |
| Berlin (Ber) | 13°20´E | 52°20´N | 6 | VII/VIII.00* |
| Italy | | | | |
| Verona (Ital1) | 10°60´E | 45°30´N | 11 | 25.VIII.02 |
| Mantova (Ital2) | 10°50´E | 45°10´N | 16 | 13.VIII.02 |
| Spain (Esp) | 00°40´E | 41°40´N | 18 | VI.02 |
| Norway (Nor) | 05°40´E | 58°50´N | 24 | 24.VIII.02 |
| United Kingdom (UK) | 00°40´W | 51°10´N | 23 | VI/VIII.00*<br>22. VII.02 |
| France (Fra) | 01°30 | 43°30`N | 5 | 19.VI.02 |
| total | | | 182 | |

*= Malaise Trap catches

### 2.2.2 DNA extraction, cloning and PCR-RFLP-analysis

6-25 individuals were analysed from each locality. DNA was extracted using a modification of the method originally developed by Aljanabi & Martinez (1997). For this, the thorax from an individual was homogenized in 400 µl extraction buffer (0.4 M NaCl, 10 mM Tris-HCl, pH 8.0, and 2 mM EDTA, pH 8.0), 40 µl 20% SDS, and 8 µl proteinase K (20mg/ml) and then well mixed. After incubation at 65°C for 60 min, 300 µl 6 M NaCl was added and vortexed for 30 s. The tubes were centrifuged for 30 min at 10,000 g and thereafter the supernatant was transferred to a new tube and centrifuged for further 10 min at 10,000 g. The resultant supernatant was then transferred to a new tube, mixed with an equal volume of –20°C cold isopropanol, and centrifuged for final 20 min at 4°C and 10,000 g. Subsequently the pellet was washed two times with 70% ethanol, dried and finally re-suspended in RNase (0.01 mg/ml) in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.6) and aliquots were frozen at -20°C.

The A+T-rich region (i.e., control region) of mtDNA was first amplified in PCR reactions with low specificity (annealing temperature for the first 4 cycles 45°C, then 33 cycles at 51°C). A primer pair located in the flanking regions of the published A+T-rich region sequence of *Lucilia eximia* (Wiedemann) (Diptera: Calliphoridae) (Lessinger & Azeredo-Espin, 2000) was designed. After cloning of amplified DNA fragments of suitable size in vectors (pGEM-T Easy, Promega; pbluescript II $Sk^-$, Stratagene) following procedures from Sambrook et al. (1989) and manufacturers protocols, respectively, these were sequenced (contract sequencing by MWG Biotech).

By means of the sequence, a specific primer pair was designed:
ATsps: 5′-CATCGTCGCGCTGTAGTT-3′ as sense primer and ATspas:
5′-CACTGTTAAAACGAGGACACCTTACA-3′ as anti-sense primer. All amplifications were performed in a Biometra T3 thermocycler. For each amplification, 3-5 µl DNA was added to a 50 µl reaction mixture containing 1x PCR buffer (Qiagen), 2.5 mM $MgCl_2$, 250

µM each of dATP, dTTP, dCTP and dGTP, 1.25 – 2.0 units of HotStar *Taq* polymerase (Qiagen) and 0.3 µM of each primer. The amplification profile consisted of one cycle of 15 min at 95°C (activation of HotStar *Taq* polymerase), 37 cycles of 30 s at 94°C, 30 s at 64°C, 90 s at 72°C and one cycle of 10 min at 72°C. PCR products were then stored at 4°C.

The second nuclear DNA sequence is a derivative of the Hox genes. The fragment used was sequenced by the Max-Planck-Institut für biophysikalische Chemie in Göttingen, Germany, and was provided by Dr. U. Schmidt-Ott. The specific primer pair used for this region was Spez1s: 5´-GTCCTCCGACTATCTTTCTAA-3´ and Spez1as: 5´-TACAATTCTAACAATCGGTGA-3´. For this primer pair the same PCR protocol as for the A+T-rich region was used, except for the cycling parameters which were one cycle of 15 min at 95°C, 37 cycles of 30 s at 94°C, 30 s at 52.5°C, 60 s at 72°C and one cycle of 10 min at 72°C. PCR products were then stored at 4°C.

After amplification, aliquots of the products were cleaved according to the manufactures protocols with the following restriction enzymes: For *zen*-region: BsrI, HinfI, PaeR7I and PstI; for A+T-rich region: AccI, BsaHI, HhaI and TaqαI (MBI Fermentas and NEB).

PCR products were separated by electrophoresis on 1% Agarose gels and restriction fragments on 2-2.5% agarose gels, both in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.4) and stained with ethidium bromide. A standard DNA marker (100 bp DNA ladder, MBI Fermentas) was included on each gel.

### 2.2.3 Data analysis

For all banding patterns of the different restriction enzymes of both DNA regions tested a 0/1 character code, i.e. the haplotype, was formulated for each individual. Haplotype diversity (h) and nucleotide diversity within populations were calculated using REAP 4.0 (McElroy et al., 1992). Haplotype diversity is equivalent to gene diversity and describes the average proportion of heterozygotes per locus in a randomly mating population. Nucleotide diversity is the average number of nucleotide substitutions within a population (Nei & Tajima, 1981; Nei, 1987). The genetic structure of the populations was analysed by Analysis of Molecular Variance (AMOVA) using the Arlequin 2.001 software (Schneider et al., 2001). AMOVA estimates the amount of genetic variation attributable to genetic differentiation among self-defined groups ($\Phi_{CT}$), among populations within groups ($\Phi_{SC}$), and among populations relative to the total sample ($\Phi_{ST}$). These $\Phi$ values, whose significance was tested using 16,002 non-parametric permutations, are analogous to conventional *F*-statistics (Excoffier et al., 1992). Slatkin's linearized $F_{ST}$'s (Slatkin, 1995) between pairs of populations were used as input variables for a Principal Coordinates Analysis (Gower, 1966) using the DistPCoA program (Legendre & Anderson, 1998). Correction for negative eigenvalues was performed using the Lingoes method (Lingoes, 1971). Effective migration rates (Nm) were calculated from $F_{ST}$ ($\Phi_{ST}$) values generated with Arlequin 2.001. *Nm* provides an estimate of the number of migrants per generation. It assumes the infinite-island model of population structure and gene flow (Wright, 1951). Although most populations probably do not conform to this assumption, it provides a useful estimation of the relative extent of gene flow. Isolation by distance was analysed by a regression of pairwise $\Phi_{ST}$ /1-$\Phi_{ST}$ against natural logarithm (ln) of geographical distances in kilometres. A modified Mantel test over 10,000 permutations as implemented in the ISOLDE program in Genepop 3.4 (Raymond & Rousset, 2003) was used to test the

null hypothesis that the two variables were independent. For both DNA regions, the relationships between haplotypes were evaluated using Nei & Li's (1979) distance between pairs of haplotypes and unrooted neighbor-joining trees (Saitou and Nei, 1987) were constructed using the Neighbor and Consense routine in the software Phylip 3.6b (Felsenstein, 2004). Support for the tree nodes was assessed by bootstrap analysis over 1000 iterations. Because Phylip 3.6b is beta version software, results were partly checked with Phylip 3.57c (Felsenstein, 1993).

## 2.3 Results

### 2.3.1 Distribution of haplotypes

PCR-amplifications of the A+T-rich region led to an approximately 1,470 bp long fragment with no detectable size differences between the populations. Using this sequence, four restriction enzymes (BsaHI, AccI, TaqαI and HhaI) were selected to obtain RFLP markers. All enzymes produced banding patterns and all cutting sites polymorphisms. In total, 12 haplotypes were found (table 2), with approximately two haplotypes per population. Unique haplotypes (in total seven) were found in five populations, three of them in the Harz-Braunlage (Germany) population (table 2). Haplotype 1 was present in all populations, and haplotypes 6, 10, 11, and 12 in two populations. Haplotypes found in two locations were not spatially aggregated but distributed over the entire sampling region (figure 1b, table 2).

PCR-amplifications of the *zen*-region yielded an approx. 1,170 bp long fragment, also with no detectable size differences between the populations. The following restriction enzymes were selected: HinfI, BsrI, PaeR7I and PstI. All enzymes cut at polymorphic sites; in total 18 haplotypes were found, with approximately five haplotypes per population (table 3). The highest numbers of unique haplotypes (in total eight) were recorded in the populations from Elm (Germany) and Norway; seven populations had no unique ones (table 3). Five haplotypes occurred once and three haplotypes more often, though only at one site. Haplotype 1 was present in all populations; haplotypes 3, 5, 7, and 8 were also rather common, and occurred in more than half of the populations sampled (figure 1a, table 3).

**Table 2:** A+T-rich region haplotype distribution and number in all populations, total number of haplotypes and unique haplotypes per population.

| Haplotype / Sites* | Hel | Hrz1 | Hrz2 | S-H | Elm | Mue | Ber | Ital1 | Ital2 | Esp | Nor | UK | Fra |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 5 | 18 | 14 | 5 | 18 | 8 | 6 | 10 | 15 | 17 | 23 | 22 | 5 |
| 2 | | | | | | | | | | | | 1 | |
| 3 | 2 | | | | | | | | | | | | |
| 4 | | 1 | | | | | | | | | | | |
| 5 | | | | | | | | | | 1 | | | |
| 6 | 1 | | 1 | | | | | | | | | | |
| 7 | | 1 | | | | | | | | | | | |
| 8 | | 1 | | | | | | | | | | | |
| 9 | | | | | | | | | | | 1 | | |
| 10 | 1 | | | 1 | | | | | | | | | |
| 11 | 1 | | | 1 | | | | | | | | | |
| 12 | | | | | | | | 1 | 1 | | | | |
| Haplotype numbers | 5 | 4 | 2 | 3 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 1 |
| Unique haplotypes | 1 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |

*For abbreviations see table 1.

**Table 3:** *zen*-region haplotype distribution and number in all populations, total number of haplotypes and unique haplotypes per population.

| Haplotype / Sites* | Hel | Hrz1 | Hrz2 | S-H | Elm | Mue | Ber | Ital1 | Ital2 | Esp | Nor | UK | Fra |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 5 | 9 | 3 | 8 | 3 | 1 | 6 | 6 | 7 | 4 | 16 | 2 |
| 2 | | | 2 | | | | | | | | | | |
| 3 | | 4 | 1 | 1 | 1 | | | | 1 | 1 | 1 | 2 | |
| 4 | 1 | 2 | 1 | | | 1 | | | 1 | | | | |
| 5 | 1 | | 2 | 1 | 1 | 1 | | | | | | 1 | 1 |
| 6 | | | | | | | | | | 1 | | 2 | |
| 7 | 5 | 2 | | 1 | | | | 5 | 2 | 6 | 8 | 1 | |
| 8 | 1 | 3 | | | 1 | 2 | 3 | | 3 | 2 | 2 | | 2 |
| 9 | | 4 | | | 3 | | 2 | | 1 | 1 | 2 | | |
| 10 | | | | | | | | | 2 | | 3 | | |
| 11 | | | | | 3 | | | | | | | | |
| 12 | | | | | | | | | | | 1 | | |
| 13 | | | | | | | | | | | 2 | | |
| 14 | | | | | | 1 | | | | | 1 | | |
| 15 | | | | 1 | | | | | | | | | |
| 16 | | 1 | | | | | | | | | | | |
| 17 | | | | | | | | | | | | 1 | |
| 18 | | | | | 1 | | | | | | | | |
| Haplotype number | 5 | 7 | 5 | 5 | 7 | 5 | 3 | 2 | 7 | 6 | 9 | 6 | 3 |
| Unique haplotypes | 0 | 1 | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 |

*For abbreviations see table 1.

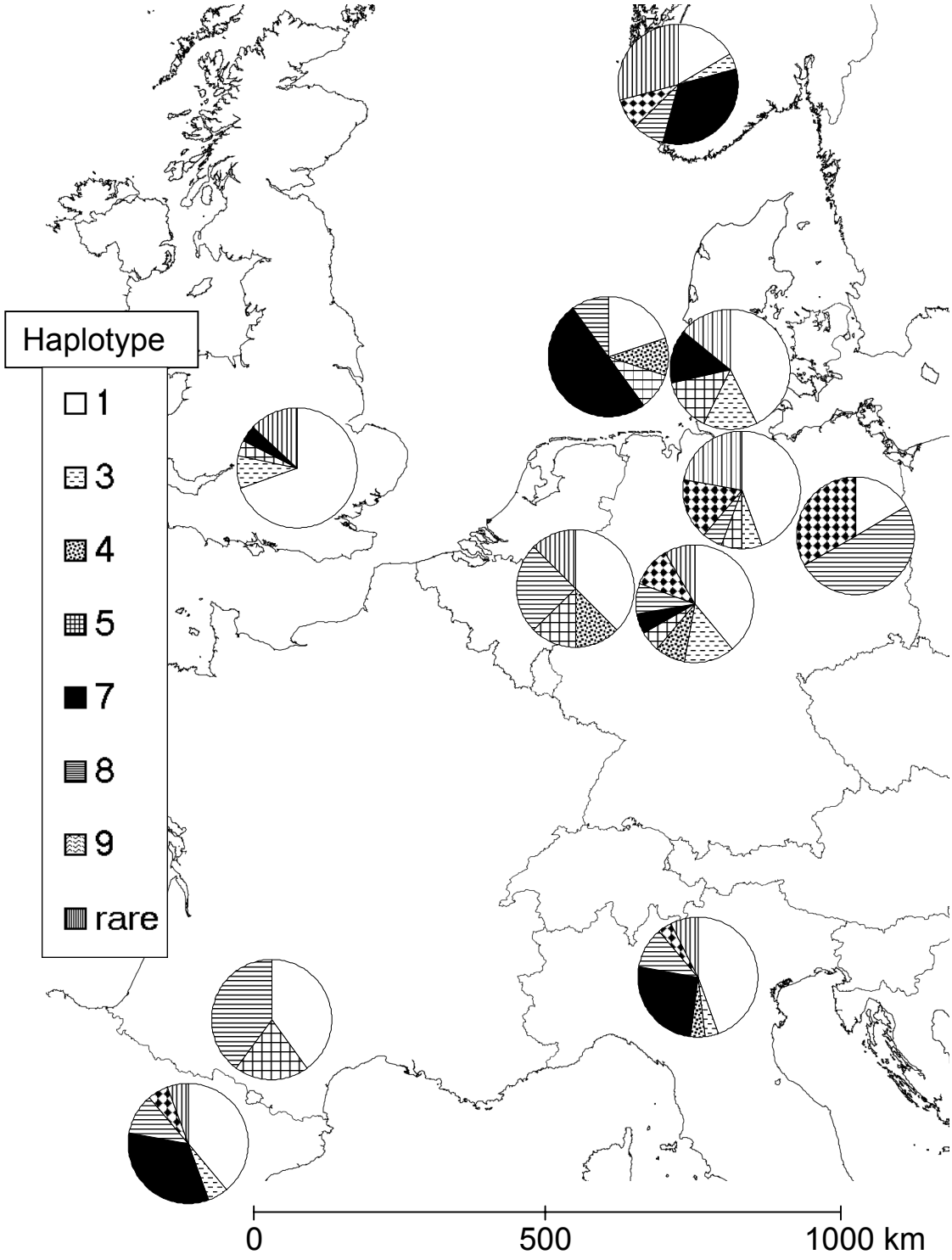


**Figure 1a:** Distribution of *Episyrphus balteatus zen*-haplotypes in Europe. For both Harz and Italy the two sites were combined in one graph, respectively. Rare haplotypes (occurring in less than three populations) were also pooled.

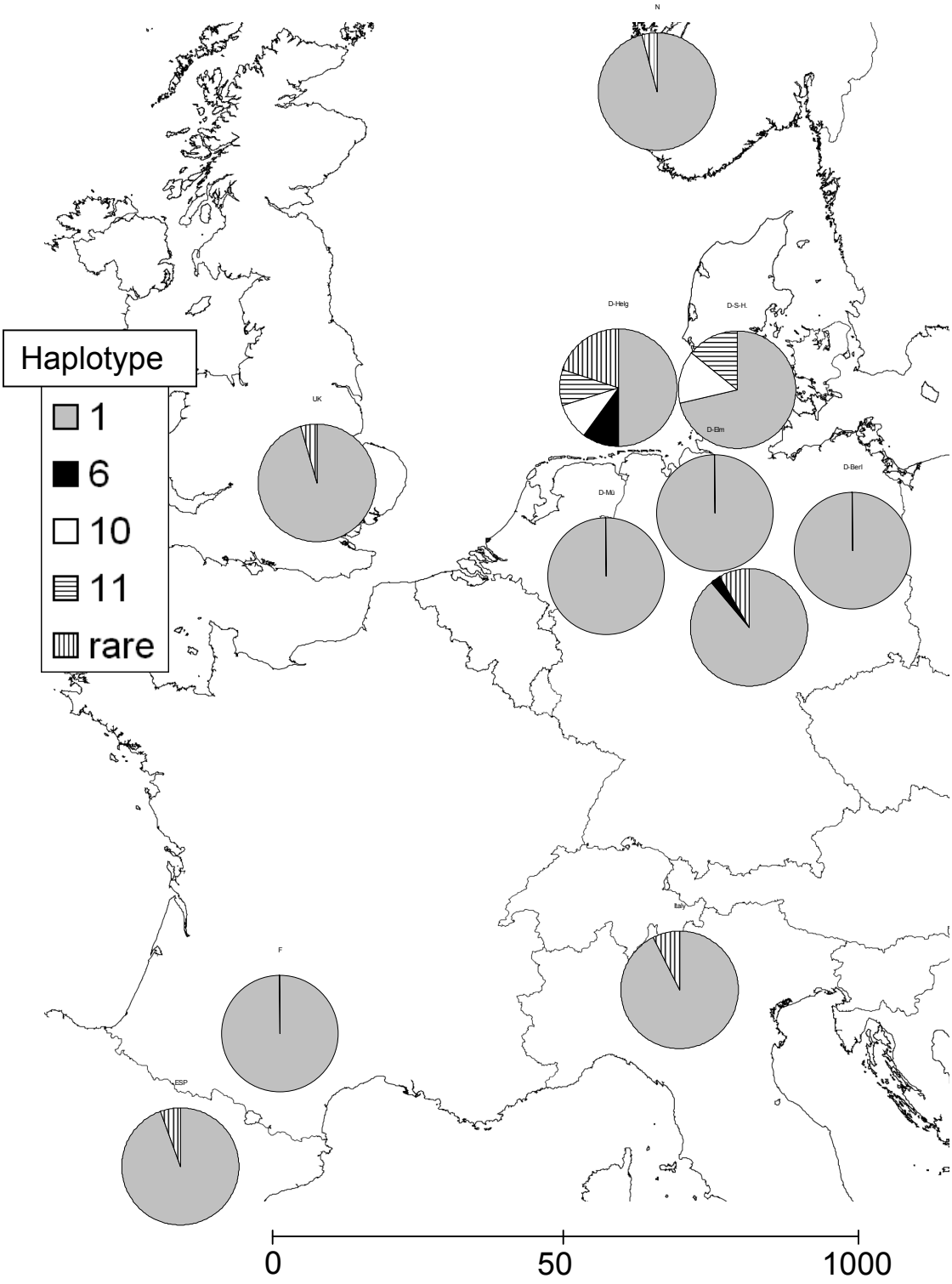


**Figure 1b:** Distribution of *Episyrphus balteatus* AT-haplotypes in Europe. For both Harz and Italy the two sites were combined in one graph, respectively. Rare haplotypes (occurring in only one population) were also pooled.

### 2.3.2 AMOVA and Φ-statistics

The AMOVA results for the A+T-rich and *zen*-region are summarized in tables 4 and 5, respectively. In both cases, the populations were partitioned into three groups, one containing the northern populations (i.e., Norway, and the two German sites in Schleswig-Holstein and Helgoland), one the southern populations (i.e., France, Italy, Spain), the third the remaining populations. In the A+T-rich region, > 99% of the genetic variation appeared as individual variation within populations, with almost no variation between populations or the three groups. Conversely, in the *zen*-region with approximately 12% of the genetic variation, considerably more variation was found between the populations, although with ca. 88% of genetic variation most of the variation appeared within the populations. Other groupings showed the same variation patterns (results not shown). The fixation indices $\Phi_{ST}$, 0.00469 ($P = 0.40864$) and 0.121 ($P < 0.0001$) for the A+T-rich and *zen*-region, respectively, also indicated low genetic differentiation between populations sampled.

**Table 4:** Analysis of molecular variance (AMOVA) using A+T-rich region haplotypes.

| Source of Variation | d.f. | Sum of Squares | Variance Components | Percentage of Variation | Φ-Statistics (Fixation Indices) |
|---|---|---|---|---|---|
| Among groups | 2 | 0.693 | 0.0026 | 1.17 | -0.0071 [a] |
| Among populations within groups | 10 | 2.026 | -0.00158 | -0.70 | 0.0047 [b] |
| Within populations | 169 | 37.76 | 0.2234 | 99.53 | 0.0117 [c] |
| Total | 181 | 40.48 | 0.2245 | | |

Probability (16002 permutations)

[a] 0.4908 ± 0.0040; [b] 0.4086 ± 0.0036; [c] 0.0596 ± 0.0019

**Table 5:** Analysis of molecular variance (AMOVA) using *zen*-region haplotypes.

| Source of Variation | d.f. | Sum of Squares | Percentage of Variation | Variance Components | Φ-Statistics (Fixation Indices) |
|---|---|---|---|---|---|
| Among groups | 2 | 6.542 | 0.12 | 0.0014 | 0.1199 [a] |
| Among populations within groups | 10 | 27.44 | 11.98 | 0.1336 | 0.1210 [b] |
| Within populations | 169 | 165.66 | 87.90 | 0.9802 | 0.0012 [c] |
| Total | 181 | 199.64 | | 0.2255 | |

Probability (16002 permutations)

[a] 0.0000 ± 0.0000; [b] 0.0000 ± 0.0000; [c] 0.4010 ± 0.0035

### 2.3.3 Diversity

Within populations, haplotype and nucleotide diversities of the A+T-rich region indicated distinct differences between such populations. Thus in the populations from Helgoland and Schleswig-Holstein (both Germany) diversity was high, whereas it was low or even equal to zero in all other populations (table 6).

In the *zen*-region, haplotype and nucleotide diversities within populations were substantially higher than in the A+T-rich region (table 6). The highest haplotype diversities were found in populations from Norway, Muenster and Harz-Braunlage (both Germany), and Verona (Italy), whereas populations from Elm (Germany) and France had the highest nucleotide diversities.

**Table 6:** Haplotype and nucleotide diversity of the A+T-rich region and *zen*-region within all tested populations. For abbreviations see table 1.

| | A+T-rich region | | *zen*-region | |
|---|---|---|---|---|
| Location | Haplotype Diversity ± SE | Nucleotide Diversity | Haplotype Diversity ± SE | Nucleotide Diversity |
| Hel | 0.756 ± 0.130 | 3.667 | 0.716 ± 0.087 | 2.620 |
| Hrz1 | 0.271 ± 0.124 | 1.629 | 0.850 ± 0.021 | 2.331 |
| Hrz2 | 0.133 ± 0.112 | 0.800 | 0.616 ± 0.089 | 1.807 |
| S-H | 0.524 ± 0.209 | 5.000 | 0.791 ± 0.086 | 2.181 |
| Elm | 0.000 ± 0.000 | 0.000 | 0.756 ± 0.057 | 4.006 |
| Mue | 0.000 ± 0.000 | 0.000 | 0.800 ± 0.066 | 2.711 |
| Ber | 0.000 ± 0.000 | 0.000 | 0.667 ± 0.091 | 2.897 |
| Ital1 | 0.182 ± 0.144 | 2.182 | 0.520 ± 0.038 | 3.818 |
| Ital2 | 0.125 ± 0.106 | 1.500 | 0.807 ± 0.048 | 3.816 |
| Esp | 0.111 ± 0.096 | 0.556 | 0.737 ± 0.047 | 3.542 |
| Nor | 0.083 ± 0.075 | 0.750 | 0.837 ± 0.035 | 2.430 |
| UK | 0.087 ± 0.078 | 0.174 | 0.506 ± 0.086 | 1.904 |
| Fra | 0.000 ± 0.000 | 0.000 | 0.711 ± 0.086 | 4.247 |
| mean | 0.175 | 1.25 | 0.716 | 2.947 |

### 2.3.4 Nm-estimates

Substantial levels of gene flow were found between all sampling sites, though *Nm*-estimates for the *zen*-region were smaller than those for the A+T-rich region (table 7). For the other region, populations from the two German sites in Helgoland and Schleswig-Holstein, and for the *zen*-region, the Berlin (Germany) population, had lower gene flow estimates. Most of *Nm*-estimates were >1 and many approached infinity, thus indicating panmixia.

**Table 7:** *Nm*-Estimates between all populations. *zen*-region estimates (in the lower left segment), A+T-rich region estimates (in the upper right segment); "inf" = infinite gene flow. For other abbreviations see table 1.

| | Hel | Hrz1 | Hrz2 | S-H | Elm | Mue | Ber | Ital1 | Ital2 | Esp | Nor | UK | Fra |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hel | - | 55.8 | 54.4 | inf | 5.03 | inf | inf | 16.6 | 7.00 | 6.98 | 5.60 | 7.87 | inf |
| Hrz1 | 4.58 | - | inf | inf | inf | inf | inf | inf | inf | inf | 118 | inf | inf |
| Hrz2 | 7.85 | 3.07 | - | 8.61 | 39.4 | inf | inf | inf | 217 | inf | 79.7 | 296 | inf |
| S-H | 35.0 | inf | inf | - | 2.86 | 24.0 | inf | 9.44 | 4.53 | 3.79 | 4.96 | 9.41 | inf |
| Elm | 5.93 | inf | 4.50 | inf | - | inf | inf | 10.1 | 65.5 | inf | inf | inf | inf |
| Mue | 3.96 | inf | 2.33 | inf | 18.0 | - | inf | inf | inf | inf | inf | inf | inf |
| Ber | 0.49 | 2.51 | 0.30 | 0.82 | 1.44 | 2.39 | - | inf | inf | inf | inf | inf | inf |
| Ital1 | inf | 1.98 | 3.01 | 2.15 | 1.92 | 1.19 | 0.19 | - | inf | 52.7 | inf | inf | inf |
| Ital2 | 7.77 | inf | 2.14 | 10.1 | 11.4 | inf | 2.07 | 2.17 | - | 1183 | inf | inf | inf |
| Esp | inf | 7.01 | 5.22 | 15.1 | 6.08 | 6.12 | 0.62 | 66.3 | 29.1 | - | inf | inf | inf |
| Nor | 7.35 | 6.05 | 1.46 | 3.77 | 4.80 | 7.64 | 1.80 | 2.15 | inf | 8.64 | - | inf | inf |
| UK | 6.35 | 2.78 | 9143 | 9.37 | 2.47 | 1.89 | 0.26 | 4.08 | 2.01 | 6.38 | 1.35 | - | inf |
| Fra | 4.07 | inf | 2.36 | inf | inf | inf | 3.97 | 0.94 | inf | 7.46 | 14.3 | 1.52 | - |

### 2.3.5 Isolation by distance

Isolation by distance will generate positive correlations between pairwise geographic distances and estimates of $\Phi_{ST}$ /1-$\Phi_{ST}$ (genetic distance). When plotting $\Phi_{ST}$ /1-$\Phi_{ST}$ against

geographical distance of all *E. balteatus* populations, no positive correlations were found (figures 2a & b). For both regions, $\Phi_{ST}$ /1-$\Phi_{ST}$ were independent of geographical distances (A+T-rich region: Spearman's $P$ = 0.67820; *zen*-region: Spearman's $P$ = 0.69060; Mantel test with 10,000 permutations). Thus, no evidence for an isolation by distance effect was found.

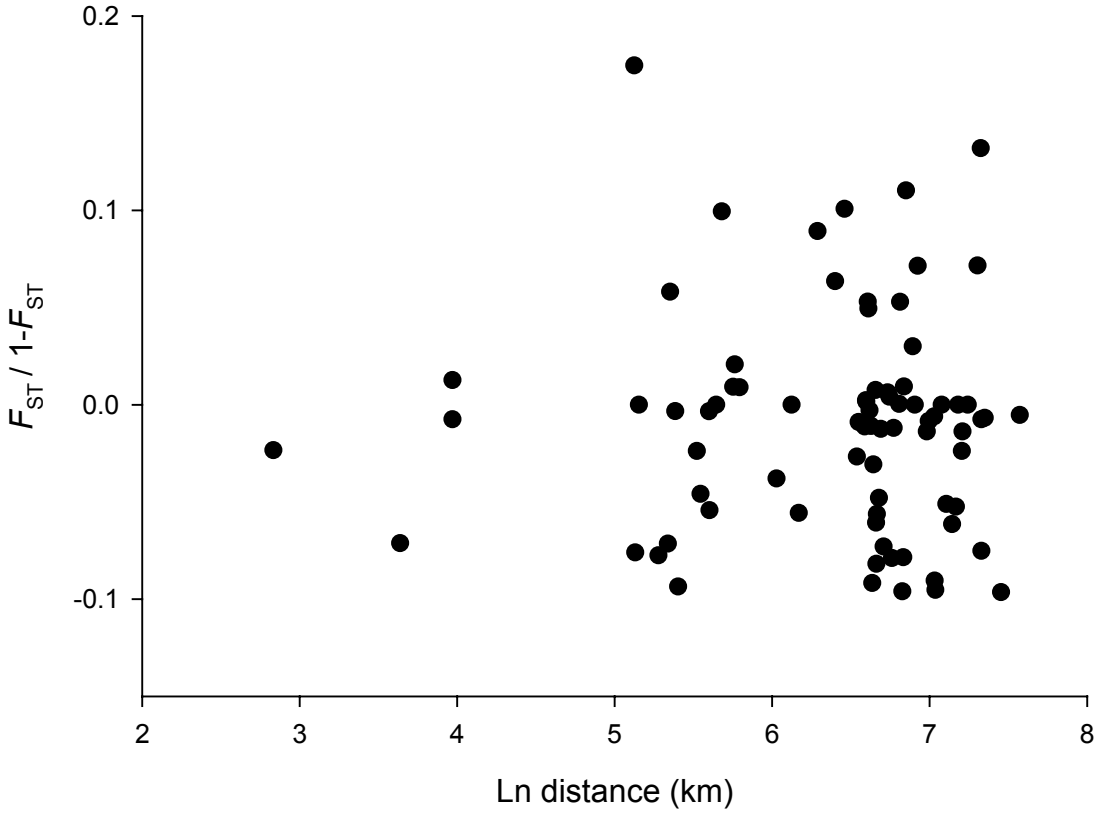


**Figure 2a:** Scatterplot of pairwise $F_{ST}$ /1-$F_{ST}$ and ln of pairwise geographical distance between all populations for the A+T-rich region (a = -0.00787, b = -0.000357, $r^2$ = 0.000035).

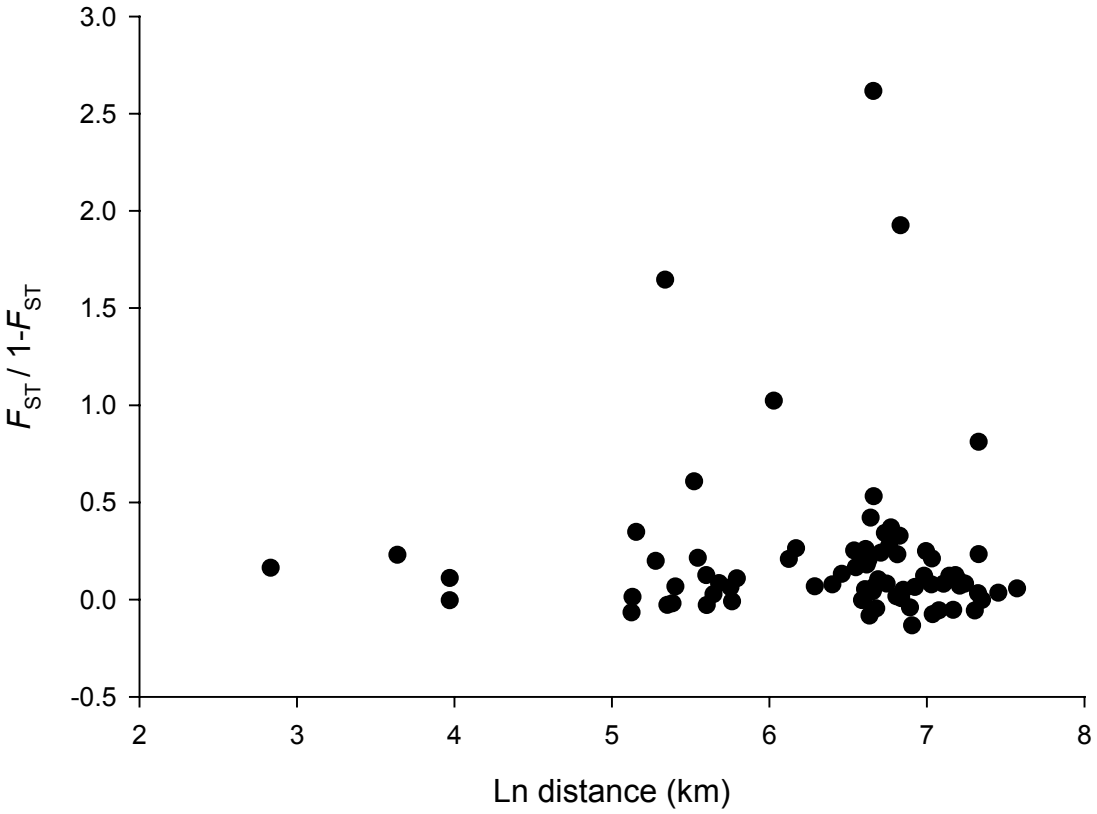


**Figure 2b:** Scatterplot of pairwise $F_{ST}$ /1-$F_{ST}$ and ln of pairwise geographical distance between all populations for the *zen*-region (a = 0.24840, b = -0.00564, $r^2$ = 0.00015).

### 2.3.6 Principal coordinates analysis

Figures 3a & b show the Principal Coordinates Analysis scattergrams derived from all *E. balteatus* populations. As shown no clear patterns of relationship between populations were detected. In the A+T-rich region, only populations from the two German sites in Schleswig-Holstein and Helgoland were substantially outside the main cluster. In the *zen*-region, populations from UK and Harz-Kamschlacken (Germany) formed a second cluster, and those from Verona (Italy), Berlin (Germany) and Norway were considerably outside the two clusters (figure 3b).

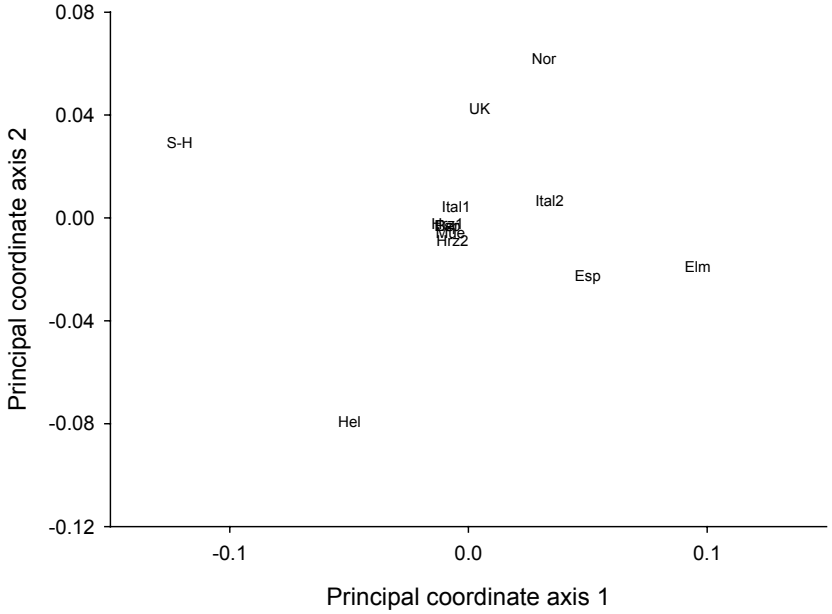


**Figure 3a:** Principal coordinates analysis of Slatkin's $F_{ST}$ distances of the A+T-rich region for all analysed populations (the dot accumulation at zero coordinates contains Harz-1, Harz-2, France, Muenster and Berlin). For abbreviations see table 1.

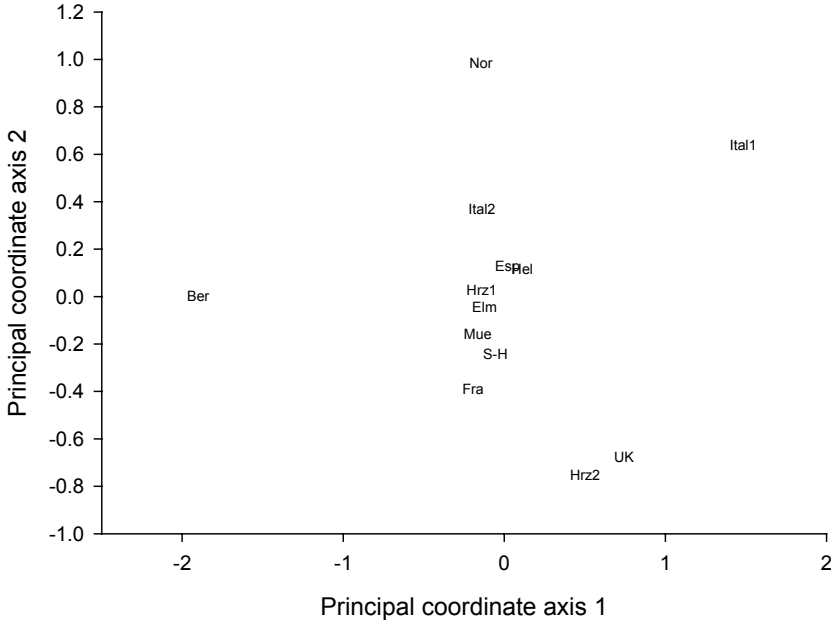


**Figure 3b:** Principal coordinates analysis of Slatkin's $F_{ST}$ distances of the *zen*-region for all analysed populations. For abbreviations see table 1.

### 2.3.7 Haplotype relations

Neighbor-Joining trees showing the relationships between haplotypes based on Nei & Li´s distance are presented in figure 4a and 4b. Generally for both DNA regions haplotypes there were no geographic patterns or genetic lineages visible and the bootstrap support for most of the branches is weak. In the A+T-rich region only the haplotypes 2 and 10 (UK (2) & Helgoland, Schleswig-Holstein (10)) form a distinct group and are geographically somewhat neighbouring. In *zen*-region only the haplotypes 2 and 11 (Harz-2 and Elm) form a separated group and they are also connected to some degree. Trees based on maximum parsimony and UPGMA analysis revealed similar trees.

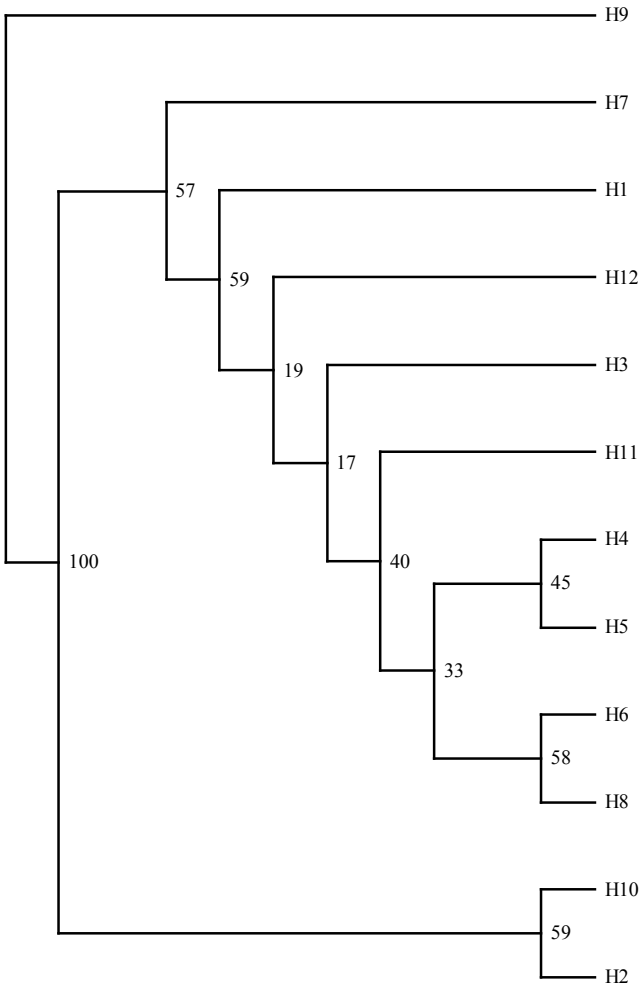


**Figure 4a:** Unrooted neigbor-joining tree using Nei & Li´s genetic distance between pairs of A+T-rich region haplotypes. H1 - H12 are haplotype numbers (see table 2). Numbers above branchings indicate bootstrap support.

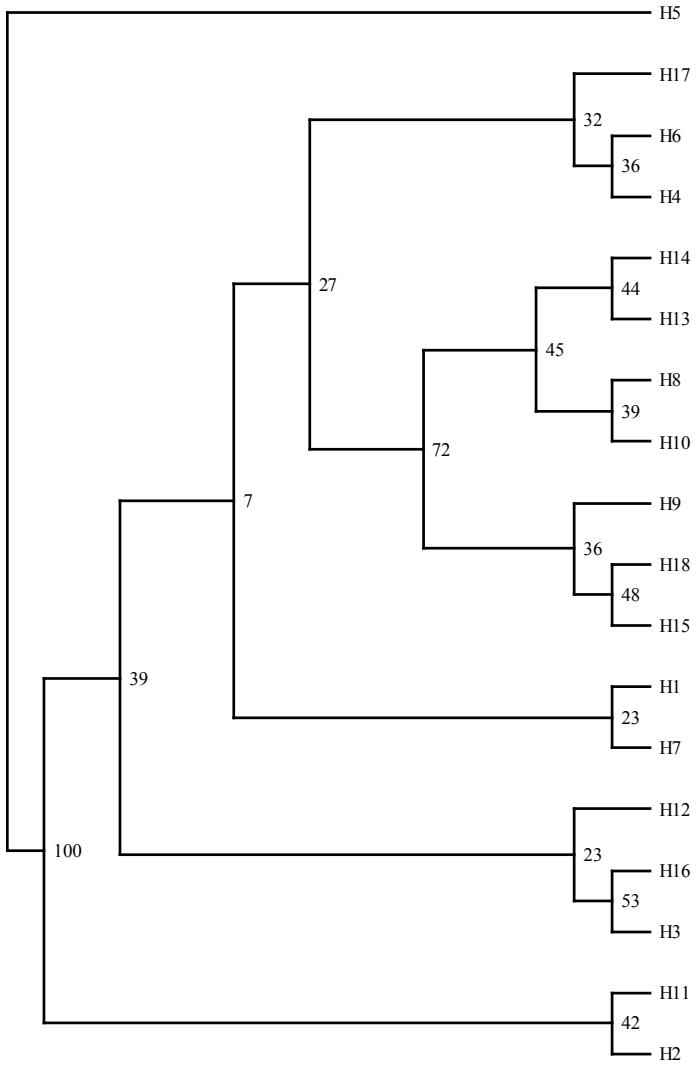


**Figure 4b:** Unrooted neigbor-joining tree using Nei & Li´s genetic distance between pairs of zen-region haplotypes. H1 - H18 are haplotype numbers (see table 3). Numbers above branchings indicate bootstrap support.

## 2.4 Discussion

Results of this study strongly suggest that European populations of *E. balteatus* are connected to a large extent and form one large panmictic population. These findings support the hypothesis of seasonal migrations in *E. balteatus* as suggested by several authors (e.g., Aubert et al., 1976; Verlinden & DeCleer, 1987; Gatter & Schmid, 1990; Torp, 1994; Sullivan & Sutherland, 2000). This hypothesis was until now primarily based on two long-term studies of mass movements of *E. balteatus* and other hoverfly species along passes in the Schwäbische Alp (South-Western Germany) and the Swiss Alps and numerous occasional flight observations across Europe (Aubert et al., 1976; Gatter & Schmid, 1990 and references therein). These observations were interpreted as southbound migrations heading to Mediterranean regions for hibernation. Moreover, physiological experiments, revealing low levels of cold hardiness in *E. balteatus* (Hart & Bale, 1997b), suggested an overwintering in warmer climates.

However to date, northbound migration of *E. balteatus* in spring from the Mediterranean to central and northern Europe has never been observed, although it is essential for this hypothesis. The unfavourable climatic conditions as well as the periodically depletion of food sources for adults and larvae of *E. balteatus* during the hot summer months in the Mediterranean are quoted as reasons for such a migratory pattern (Gatter & Schmid, 1990). The results of this study suggest that in the framework of the seasonal migration hypothesis, northbound migrations are very likely to occur, because the genetic diversity of the sampled *E. balteatus* populations turned out to be high. Assuming that the high densities of *E. balteatus* frequently recorded during the summer months in central and northern Europe derive only from overwintering individuals in these areas, and considering the extremely low numbers of hibernating residents (findings of residents in

*E. balteatus* are extremely rare) would consequently result in bottleneck effects with largely reduced genetic diversity (Nei et al., 1975; Hedrick, 2000).

Even so, it is still uncertain how regular these migrations are. For example, Salveter (1996) stressed that during the period of southward migrations of hoverflies, the vegetation in the Mediterranean is still very dry, thus providing insufficient food for the adults and the aphidophagous larvae of *E. balteatus*. Hence, a more facultative or irregular migration pattern might occur (e.g., in the sense of Svensson & Janzon, 1984), only triggered by abiotic (e.g., weather) or biotic conditions (e.g., food resources). This would not exclude the probability that *E. balteatus* migrates over large distances, leading to the observed highly connected populations, with low genetic distances, high gene flow, and high genetic diversity.

Physical barriers to migration such as mountains or seas and isolation by distance effects can strongly influence gene flow even in mobile insects (e.g., Gimnig et al., 1999). However, in European populations of *E. balteatus*, no such effects were recorded as shown by the lack of population subdivision, the low genetic distances between sampled populations, the very high gene flow rates, and the complete lack of isolation by distance even between populations separated by mountains (Swiss Alps, Pyrenees) and the sea (North Sea).

At present, studies on the population genetics of mobile hoverflies covering broad geographical regions are lacking. So far, work has focussed on genera with patchy distribution and non-migratory behaviour such as *Portevinia* or *Cheilosia* spp. (Diptera: Syrphidae) (Wynne, 2001; Milankov et al., 2002; Ludoski et al., 2003). In *E. balteatus,* Sullivan & Sutherland (2000) studied morphological patterns (i.e. asymmetry measures) between specimens from nine European sites. They found considerable homogeneity in most morphological measures, except for total body size, which led to the assumption that, only one population, from the island of Madeira (> 450 km from the African continent)

might be isolated. Despite numerous observations of incoming migrants along coast lines (e.g., Svensson & Janzon, 1984; Gatter & Schmid, 1990), it is presently unclear to what extent *E. balteatus* is capable of migrating offshore, as it is not known if or how often it needs 'refuelling' and resting periods during migration. In addition, in this study, *E. balteatus* populations from islands (UK and Helgoland, Germany), showed a slightly increased similarity and a higher genetic distance compared to populations from other sites. But these differences are not significant, and it is unlikely that this is an indication for separation from other populations. Very few (<10) migrants are needed each generation to maintain genetic homogeneity (Hartl, 1988). Nm values greater than 4 are a sign that gene flow has a major role in shaping the genetic structure of a population (Schnabel & Hamrick, 1990). This number of migrants can likely cross the Channel and the North Sea as indicated by several observations (Gatter & Schmid, 1990).

Similar or lower levels of genetic differentiation have been found over wide spatial scales in other mobile insect species such as monarch butterflies, *Danaus plexippus* (Linnaeus) (Lepidoptera: Nymphalidae) (Brower & Boyce, 1991), stable flies, *Stomoxys calcitrans* (Linnaeus) (Diptera: Muscidae) (Szalanski, 1995), the planthoppers *Nilaparvata lugens* (Stål) and *Sogatella furcifera* Horvath (both Hemiptera: Delphacidae) (Mun et al., 1999), dragonflies, *Anax junius* (Drury) (Odonata: Aeshnidae) (Freeland et al., 2003) or aphids, *Sitobion avenae* (Fabricius) (Hemiptera: Aphididae) (Llewellyn et al., 2003). Hence, this seems to be a relatively typical phenomenon, at least if migration and gene flow are linked, which is not always so (Slatkin, 1985). In *E. balteatus* populations, reproduction is proven in northern and southern areas of Europe (e.g., Tizado-Morales et al., 1991; Steenis et al., 2001), but it was previously not known if, and to what extent, migrants contribute to the local gene pool. Our findings that a number of common haplotypes are distributed over the entire study area in Europe and the strong genetic

similarity between these populations imply that long distance migration determines the genetic structure and that local gene pools cannot be separated.

The observed high genetic diversity within the *zen*-region in most *E. balteatus* populations is particularly remarkable. Often low genetic diversity is connected with lack of genetic structuring and high diversity with high levels of structuring (e.g., Brower & Boyce, 1991; Szalanski, 1995; Gimnig *et al*, 1999), whereas high diversity and low geographic structuring is rarely found (e.g., Mun et al., 1999; Freeland et al., 2003). A general explanation for the high genetic diversity and the widespread distribution of common haplotypes in most populations may be an adaptation of *E. balteatus* to a broad range of environmental conditions (e.g., temperature, humidity, photoperiod) and biotic factors (e.g., prey species and distribution, food plants, natural enemies). All developmental stages of a species have to be adapted to this array of selection pressures, and a high genetic diversity can increase fitness and the ability to cope with these challenges (Hansson & Westerberg, 2002), although direct correlations are often weak (David, 1998).

In this study, the *zen*-region showed larger genetic diversity and revealed more population subdivision compared with the A+T-rich region. Yet, the latter is one of the most variable mtDNA regions and evolves much faster than coding nDNA regions (Simon et al., 1994; Parker et al., 1998; Ballard & Whitlock, 2004). However, variability can vary to a large extent between species, and species with low A+T-rich region variability are known (e.g., Taylor et al., 1993; Dueñas et al., 2002). There are three possible explanations for the observed lower genetic variability in the A+T-rich region: (i) based on haploid structure and maternal inheritance, the mtDNA effective population size is only a fourth of that of nuclear DNA (Zhang & Hewitt, 2003), which increases the power of genetic drift, leading to a decline in within-population heterozygosity. Consequently, migration influences genetic structure more strongly by easier fixation of haplotype

lineages over all populations. The effective population size of *E. balteatus* is likely to be considerably smaller than one might expect based on the high numbers frequently found during the summer, since the lowest population density during the autumn and winter months largely determines the overall effective population size (Hedrick, 2000); (ii) for example in *Drosophila* spp. (Diptera: Drosophilidae), the A+T-rich region consists of three domains with different variability (Brehm et al., 2001), making it possible that a more conserved domain of this region was sequenced and then analysed; (iii) the evolutionary rate and variability of the *zen*-region is unknown; thus its variability possibly exceeds that of the mtDNA region. The fundamental functional changes of this region during its evolution might be indicative of this (Hughes & Kaufman, 2002; Stauber et al., 2002).

For pest management purposes, it would be disadvantageous if the large summer populations of *E. balteatus* mainly develop from immigrants. This is because in northern Europe, these individuals often arrive too late for sufficient control of for example, cereal aphids. This lack of synchronisation between hoverflies and cereal aphids is believed to be one of the main reasons for frequent pest outbreaks in northern Germany (Tenhumberg & Poehling, 1995), whereas in southern parts of Germany, aphids are most often under the control of natural enemies owing to the earlier arrival of hoverflies and other beneficial insects.

# 3. Local Overwintering of *Episyrphus balteatus*

## 3.1 Introduction

In temperate and cold climates, insect life cycles are correlated closely with seasonal changes. This ensures that insect development and reproduction are restricted to periods of the year when weather conditions are favourable and there are suitable sources of food. Migration and diapause are two of the mechanisms that insects use to ensure that their offspring are not subjected to adverse conditions (see Tauber et al., 1986). Diapause is a distinct physiological stage that is regulated by neuroendocrine processes and that is generally induced by combinations of changes in photoperiod and/or temperature. Usually, insects pass through a sensitive period before entering diapause, which allows them the time necessary to adapt to the changing conditions (Tauber et al., 1986). Reproductive diapause in adult insects is usually characterized by a delay in the development of the gonads and an accumulation of energy reserves (e.g., the fat body).

It was assumed for a long time that only the females of *Episyrphus balteatus* (DeGeer) were able to overwinter in temperate climates and that these individuals then gave rise to the first generation of hoverflies in the spring. However, it is now known that large numbers of *E. balteatus* migrate south during the autumn period. Therefore, it has been suggested that a comparable migration to the north helps to re-establish *E. balteatus* populations in northern Europe during the spring. There are many records to show that some hoverfly adults are active so early in the spring that they could not possibly be long-distance migrants. Nevertheless, although some adults remain active late into the autumn (e.g., Krause & Poehling, 1996; Salveter, 1996; P Hondelmann, unpubl.), few authors have managed to find females in overwintering sites (e.g., Kula, 1982; Wolff, 1996).

Hart & Bale (1997b) found that the cold-hardiness in UK populations of *E. balteatus* was low and that the few adults that managed to survive the winter were believed to use artificial hibernation sites (e.g., overwintering in greenhouses). Other studies have suggested that *E. balteatus* migrates in large numbers during late summer and autumn to the Mediterranean area and only return to central and northern Europe during the following year (Aubert et al., 1976; Gatter & Schmid, 1990; Kehlmaier& Martínez de Murguía, 2004; Hondelmann et al., 2005).

The synchronisation of *E. balteatus* populations in the spring with the food of its larvae, mainly aphids, is of special importance. This is because early oviposition by the hoverflies at the time the aphid populations are beginning to build-up is regarded as crucial for regulating cereal aphid numbers (Ohnesorge & Schier, 1989; Poehling et al., 1991; Corbett, 1998). Long-term studies have suggested that fluctuations in cereal aphid numbers in different parts of Germany may be associated with the timing of *E. balteatus* populations in the spring. In spring in southern Germany, *E. balteatus* was observed colonizing cereal fields in sufficient numbers to prevent aphid outbreaks. In contrast, in more northern areas, the hoverflies often arrived too late to be effective in reducing aphid numbers (Poehling et al., 1991; Tenhumberg & Poehling, 1995; Krause, 1997). It is suggested that southern areas of Germany are probably colonized earlier by hoverflies that migrate from the Mediterranean area. In addition, in southern Germany more hoverflies could survive the winter, as the more diverse landscape appears to provide more potential overwintering sites. In spring in southern Germany, food is available for the hoverfly adults from early-flowering plants and shrubs and for the hoverfly larvae from the first aphid colonizers of several plants. In northern Germany, however, such important winter and spring habitats are no longer available, due to changes to the landscape that have been made during the last few decades (e.g., Ohnesorge & Schier, 1989; Tenhumberg & Poehling, 1995).

This paper deals with the physiological adaptations that enable the hoverfly *E. balteatus* to overwinter in northern Germany. The main aim of the research was to study the induction and termination of diapause under controlled laboratory conditions. We were particularly interested in studying how changes in photoperiod affected development of the different stages of *E. balteatus*, in determining the sensitive stages for diapause induction, and in the factors that controlled the termination of diapause. All of the earlier studies (Dušek & Láska, 1974; Krause & Poehling, 1996; Hart & Bale, 1997b) appeared to show that only the females of this hoverfly overwinter in colder climates. Therefore, we concentrated our efforts on the females. We also did a semi-field experiment, to confirm the laboratory results and to determine whether this insect could survive the conditions found during winter in Hannover.

## 3.2 Material and methods

### 3.2.1 Diapause

*3.2.1.1 Insect cultures*

Adults of *E. balteatus* were collected from fields near to the Institute of Plant Diseases and Plant Protection in Hannover, Germany (52°23` N, 9°44` E) to start the laboratory colony of this hoverfly. Regularly, new individuals were collected from the field and introduced into the colony to prevent inbreeding depression. The colonies were kept in a room maintained at 21 ± 2°C, L15.5:D8.5, ca. 3500 lux, and 40-60% r.h. The hoverfly adults were kept in flight cages (52 x 60 x 40 cm) that consisted of a white plastic frame covered by nylon gauze, and a transparent perspex lid. The adults were provided with pollen (ground pollen mix from bee pollen balls) and sucrose. Water was provided in Petri dishes on moist tissue paper. Twice a week, bean plants (*Vicia faba* L. cv. Hangdown) that were infested with *Aphis fabae* Scopoli (Homoptera: Aphididae), were placed into each cage to stimulate the hoverflies to lay eggs.

The hoverfly larvae were kept in transparent plastic cages (13 x 15 x 5 cm) and provided, ad libitum, with colonies of the aphids *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae) and *A. fabae*. To synchronize the age of the hoverflies for experimental purposes, cohorts of larvae were reared from eggs that had been laid during a 3-h period. The test larvae were reared individually, from the second instar ($L_2$) onwards, in transparent plastic Petri dishes, 9 cm in diameter. These Petri dishes were kept in climate chambers (Rubarth GmbH, Laatzen, Germany) which were maintained at about 3500 lux, at temperatures of either 15 or 20°C, and at various experimental photoperiods. The larvae were provided with colonies of the aphid *A. pisum* as food and with wet 1 $cm^2$ dishcloth, renewed twice weekly, to maintain an appropriate humidity. Adults that

developed from the various treatments were kept under the same conditions as the stock colonies of this hoverfly.

*3.2.1.2 Effects of photoperiod on development of larvae, pupae, and adults*

As preliminary experiments indicated that diapause was induced by changes in photoperiod and temperature, the induction of diapause was studied using a range of photoperiods at temperatures of 15 and 20°C. Larvae, from the second instar onwards, were reared under the following five photoregimes: L14.5:D9.5, L12.5:D11.5, L11.65:D12.35, L10.5:D13.5, and L8:D16. These regimes corresponded to the photoperiods that occurred from August to mid-November at the latitude (52°23` N) of Hannover, Germany. Larvae for the control treatment were reared under a long-day photoperiod of L15.5:D8.5. The critical photoperiod for diapause induction was expected to be within the range of photoperiods tested. The times, in days, for the larvae and pupae to develop under the various regimes were recorded. Once adults emerged, both sexes were transferred to flight cages and kept for 2 weeks, the pre-oviposition period, to allow the females sufficient time to develop their ovaries. The percentage of females with mature eggs and the size of the ovaries were determined, but only for the insects from the 20°C treatments, using a dissecting microscope fitted with a micrometer eyepiece (Leica MZ6; Leica Microsystems AG, Wetzlar, Germany). Body colour was assessed visually and the presence of sperm in the spermathecae was checked from 10 females/photoperiod, under a phase-contrast microscope (Leica DM IL). Trypan-blue staining (Sadeghi & Gilbert, 2000) was used to separate immature from mature eggs. The above methods were used as the standard for all subsequent experiments involving the measurements of ovaries.

When compared to the insects in the control treatment, female *E. balteatus* that had entered diapause had small ovaries, no mature eggs, and much larger fat bodies. In males,

only the amount of fat present could be used to indicate that the individual had entered diapause. Calculations of the critical photoperiod, at which 50% of the females had entered diapause, were based on the percentage of females without mature eggs.

*3.2.1.3 Effects of the photoperiod on total fat content*

As a further indication that the insects had entered diapause, the total fat content of male and female *Episyrphus balteatus* was determined, using a modified gravimetric method (see Folch et al., 1957; Bligh & Dyer, 1959). To do this, the insects were reared at 20°C from the second- stage ($L_2$) to the adult stage under various photoperiods. Fat was extracted from females subjected to all five test treatments plus the control treatment. In contrast, fat was extracted from males kept at only one test treatment plus the control treatment.

For analysis, freshly killed adults were weighed using a microbalance (Sartorius MC 5, Sartorius AG, Göttingen, Germany). The hoverflies were then homogenized in 5 ml chloroform/methanol (2/1, vol/vol) with 0.01% butylated hydroxytoluene on ice, using a Teflon pistil and a Branson Sonifier B15 (20 s, 240 W) (Branson Corp., Danbury, CT, USA). The macerate was mixed well for 10 min after which the undissolved residues were separated by filtration (S&S 595 ½, Schleicher & Schuell MicroScience GmbH, Dassel, Germany). The residue was washed again with 5 ml chloroform/methanol (2/1, vol/vol) and the two filtrates were then pooled, before 5 ml of a 0.88% potassium chloride solution was added to the extract and mixed well in a glass separating funnel. After the two phases had separated, the chloroform layer was transferred to a pre-weighed glass vial. The remaining polar phase was washed again with 5 ml chloroform and, after a further separation, this second fraction was added to the first chloroform fraction. The chloroform was then evaporated from the extract using a stream of nitrogen, after which the vial was

dried for 24 h at 40°C. The amount of fat extracted, determined by reweighing the vial, was then expressed as the percentage of fat per fresh weight of hoverfly.

*3.2.1.4 Sensitive stages*

To determine the insect stages in which changes in photoperiod could induce diapause, individuals of *E. balteatus* were reared at 20°C under long-day conditions (L15.5:D8.5). After reaching the required stage, that is second-instars, third-instars, pupae, and teneral adults, individuals were transferred to a climate chamber at a photoperiod of L8.5:D15.5, as this regime is known to induce diapause. Groups of the four test stages were maintained also under a constant long-day photoperiod (L15.5:D8.5) as the control treatments.

*3.2.1.5 Diapause termination*

A preliminary experiment showed that diapause could be terminated by extending the photoperiod. Therefore, we transferred 14-day-old, diapausing females, reared at 20°C under a short-day light regime (L10.5:D13.5), to photoperiods of : L15.5:D8.5, L14.5:D9.5, L12.5:D11.5, and L11.65:D12.35. The control treatment involved maintaining some of the individuals permanently in the L10.5:D13.5 regime. In addition, the termination of diapause at 15°C was tested using photoperiods: L14.5:D9.5, L12.5:D11.5, and L11.65:D12.35. The size of the ovaries and the presence of mature eggs, 2 weeks after the insects were placed under the test conditions, were used to indicate the insects in which diapause had been terminated.

*3.2.1.6 Semi-field experiments*

To confirm the laboratory findings, a semi-field experiment was done during the autumn of 1999 and the spring of 2000. Adult hoverflies (84 females and 44 males) were collected between 15 August and 16 September 1999, after the summer populations of *E. balteatus*

had reached low numbers (see Verlinden & Decleer, 1987; Gatter & Schmid, 1990; Salveter, 1996; Krause, 1997). The remaining flies were assumed to be non-migrating individuals and therefore would need to overwinter locally. The hoverflies were put into two cages, similar to those used for the laboratory stock cultures. The cages were then placed alongside a hedgerow in the grounds of the Institute and covered with a transparent roof to protect them from heavy rain. These outside cultures were provided with the same types of food and oviposition sites as the laboratory cultures. We attempted to rear adults from all of the eggs laid in these two cages. As a result, we were able to release an additional 36 females and 35 males into the cages by 19 September, 1999. In these outdoor cages, the adults were provided with three types of hiding places, namely 1) forest litter, 2) a brick with crevices and holes, and 3) a bark-covered log of oak (*Quercus* spec.) that had been drilled with holes. Although the numbers of flies that died were recorded weekly, not all flies had been accounted for by the end of the experiment. The dates for last oviposition, last pupation, and last adult to eclose were recorded, as were the dates that specific pupae died. The latter were recognized by the pupae turning a uniform dark brown colour. Hiding places were inspected for hoverflies, each week until December, by shining a torch into the various crevices. Weather data (temperature and relative air humidity) were recorded using a data logger (Tinytag, Gemini Dataloggers Ltd, Chichester, UK) placed in one of the field cages. Rainfall data were obtained from the Institute of Meteorology, University Hannover.

*3.2.1.7 Statistical analysis*

All data for regressions and ANOVA were tested for homogeneity of variance and for a normal distribution using the Levene test and box-plots. Developmental times and ovary and body size data were transformed to $\sqrt{x+3/8}$ to stabilise the variances. Count data for

regression were transformed to $\sqrt{x+0.5}$, and the 'percentage fat' data were transformed using the arcsine of their square roots (Zar, 1999). Data were analysed using one-way ANOVAs (GLM procedure), or linear regressions (SPSS, 2003). As multiple comparisons, in two of the photoperiod experiments (effects of photoperiod on size of female ovaries and fat content), we calculated all-pair contrasts using the SAS macros %SimTests and %SimIntervals. This procedure was introduced by Westfall et al. (1999) and uses a closed testing procedure from Shaffer's Logical Constraint Method, to account for logical dependencies between the hypotheses. Moreover, P-values were adjusted for multiplicity. Otherwise, the Tukey's HSD or the Tukey-Kramer test for unequal sample sizes were used (Zar, 1999; SPSS, 2003).

The critical photoperiod was estimated in two ways: a maximum likelihood estimation (± 95% fiducial limits) using the Probit procedure (PROC PROBIT; SAS Institute, 2003) for dichotomous data (1 = diapause, 0 = non-diapause). The Probit procedure fitted diapause probabilities to a set of photoperiods using three different regression models (normal, logistic, and Gompertz distribution). Model fitting was estimated using Pearson's $\chi^2$ test. The critical photoperiod was estimated also using a "direct estimation", taken directly from the graph.

For the semi-field mortality data, a stepwise multiple linear regression was done in which weekly mortality was used as the dependent variable and the weekly means of temperature, humidity, rainfall, together with the duration of the experiment as the sets of independent variables. The temperature variable was forced into the model-building process, as we assumed temperature was of major importance for winter mortality in this species. Statistical analyses were performed using either SPSS 12 (SPSS, 2003) or SAS 9.1 (SAS Institute, 2003).

### 3.2.2 Cold hardiness

*3.2.2.1 Lethal time determination*

To measure lethal times at given temperatures, a defined number of adult flies (15 males or 15 females) were placed in small plastic boxes (see below) and transferred in climate chambers (Rubarth GmbH, Laatzen, Germany) with the desired temperatures. After given time intervals, one randomly chosen box was removed and the state of the flies checked. Females were exposed to four treatments: standard conditions (control), cold acclimatising conditions, diapause inducing conditions, and a combination of cold acclimatising and diapause inducing conditions. In males, which are not supposed to undergo a diapause stage, the diapause treatments were omitted.

Flies were reared from eggs laid within 5h either under normal conditions (see chapter 3.2.1.1 for details) or under diapause inducing conditions (only females, see chapter 3.2.1 for details). Before the experiments, flies were kept for 5 days under their rearing conditions (males and females together), then sexes were separated and either kept for additional 5 days under these conditions or under acclimatisation conditions at 10°C for 3 days and 7°C for 2 days. Experimental conditions were i) control (continuous 21°C); ii) 24-336h 5°C; iii) 24-240h 0°C; iiii) 24-120h –5°C, and iiiii) 24-120h –10°C. The treatment combinations acclimatisation and the combination diapause/acclimatisation with continuous 21°C were omitted, as no relation to natural conditions exists.

As cages, boxes of the size 13.1 x 14.8 x 5.4 cm were used, they were equipped with gaze-covered holes at each side and in the lid. The bottom was covered with filter paper to absorb water and excretions. In one edge of the box, two small Petri dishes (∅ 3.5cm) with food (pollen-sugar mix) and water similar to the stock cultures were offered. In the long-run experiments water was added with a syringe if necessary. After the experiment, the animals were warmed slowly to 21°C at a rate of 1°C/min and the box was

opened in a flight cage with food and water supply. After 24h and 48h the survival was checked. For survival testing, the flies were touched with a brush and movements were assessed and considered as healthy (normal movements and/or flight) or dead (no reactions).

*3.2.2.2 Reproduction after cold treatment*

Maximum 14 randomly chosen *E. balteatus* females out of the surviving flies from the treatments "Standard" and "Acclimatization" were transferred in the stock rearing room in small flight cages (30 x 25 x 25cm) and an aphid (*Aphis fabae*) infested bean plant (*Vicia faba* cv. hangdown) was enclosed. It was taken care that the aphid density was similar on all plants used. Due to a limited number of cages, only females from six time intervals (duration of cold treatment) were tested and the flies from the two treatments were pooled in one cage (if possible 7+7). In some of the treatments, lower numbers of females and/or less time intervals were used (e.g. in the –5°C treatment). The –10°C experiment was skipped as none of the females laid any eggs. The plant was renewed on the next day and the pooled egg numbers were counted after 48h. As a control, 14 *E. balteatus* females of the control treatment (continuous 21°C, no treatment) were transferred in a cage and egg number assessed in the same way. Additionally several females were spot-checked for mating after the experiment (see chapter 3.2.1.2 for details).

*3.2.2.3 Statistical analysis*

For all mortality data, non-linear regressions were calculated. Using up to 800 iterations, sigmoidal curves (i.e., dose-response curves) were fitted to the data. To compare treatments within temperatures, Probit analyses were performed to calculate the time necessary for 50% and 95% mortality of the animals (i.e., lethal time; LT 50 and LT 95). Since Probit analysis requires model building, the quality of the model was estimated with

Pearson Goodness-of-Fit test which uses $\chi^2$-values. When the likelihood ratio is not significant then the model being tested is a good fit to the data, because this means the model is not significantly worse than the well-fitting saturated model which is 100% accurate ($P > 0.05$). Significant differences between treatments were estimated according to Payton et al. (2003) and McDonald et al. (2005), by considering differences as significant, if 95% fiducial limits did not overlap.

Egg numbers per individual and per 48h were compared using $\chi^2$-tests within a treatment (different time intervals) and between the treatments (temperatures). For data with one degree of freedom and/or frequencies below 5, the Yates' correction was applied.

### 3.2.3 Cryo-protectant analysis

The analysis of cryoprotectants was done using modified gas chromatographic methods from Fenton & Ahere (1987) and Magni et al. (1993). Gas chromatography has the advantage of simultaneous detection and quantification of all possible occurring cryo-protectants. By means of derivatizing agents, it is possible to analyse compounds (in this case sugars and polyhydroxy alcohols = polyols), which normally are not readily monitored by GC. The reaction between a silylating agent (e.g., TMSI) and sample compounds is called ether formation or trimethylsilylation and increases volatility and thermal stability, but reduces polarity of the resulting derivatives by replacing an active hydrogen by an alkysilyl group ($Si(CH_3)_3$). The reaction generally is fast, reproducible and free of side reactions.

#### *3.2.3.1 Animal rearing and preparation*

For experimental purposes, flies were obtained from eggs laid within three hours. Emerging females were either reared for two weeks under standard conditions (21°C, ca.

50% r. h. and 3500 Lux, see chapter 3.2.1.1 for details) or under diapause inducing conditions (photoperiod of 8.5:15.5 L:D) and then transferred to climate chambers (Rumed 1201 and 1101, Rubarth Apparatebau, Laatzen, Germany) with the required acclimatisation or experimental conditions (see below), control flies were transferred in a new cage and maintained in a climate chamber under constant standard conditions (see above). After the experiment was finished, the experimental animals were killed with carbon dioxide, abdomen, legs, and wings were cut off and the thorax and head rinsed in demineralised water for 15 min in order to clean the insect from gut content. After drying, the total weight of head and thorax was determined with a microbalance (Sartorius MC 5). Then these body parts were transferred in Eppendorf cups and frozen (-20°C) until further use.

*3.2.3.2 Extraction of sugars and polyols*

The cleaned body parts were suspended in 200 µl ethanol (96%) and then three minutes homogenized with a teflon pistil in an Eppendorf cup, the pistil was cleaned two times with 200 µl ethanol (96%), which was pooled with the first portion. After 15 min centrifugation at 8000g, the supernatant was then transferred in a new cup and used for analysis. For this, 200 µl of the supernatant was pipetted in a glass vial, 20 µl of the internal standard (phenyl-β-D-glucopyranoside, Fluka) was added and then for ca. 36 h dried at 40°C.

*3.2.3.3 Derivatization*

For standard substances, 20 µl of each substance (0.03 mg/ml) was pipetted in a glass vial together with 20 µl of the internal standard (phenyl-β-D-glucopyranoside, 0.05 mg/ml in double destillated water) and then dried for ca. 36h at 40°C. Then 50 µl of the silylating

agent TMSI (1-(trimethylsilyl)imidazole, Fluka) and 300 µl acetonitrile (Roth) were added and the vial immediately closed with a gas-tight cap with a Sil/PTFE-lined septum. The mixture was heated for 20 min at 70°C in a thermo-block (Techne driblock DB 3) for completion of the silylating reaction. For sample analyses, a 10-fold amount of sample fluid was used (see Animal rearing and preparation). As calibration control, standard substances were used with the concentrations 5 µg/ml and 75 µg/ml (20 µl each).

*3.2.3.4 Gas chromatography*

The separation of the substances was done using a gas chromatograph Hewlett Packard 5890 series II. The chromatograph was equipped with the following components:

- Flame ionization detector (FID)
- Autosampler HP 7673
- Fused silica capillary column SPB-1 (30 m length, 0.32 mm inner diameter, 0.25 µm film; Supelco, Bellefonte, PA, USA)
- Splitter injector

The injector temperature was 250°C, the temperature of the detector 300°C, and the starting temperature 100°C for 4.5 min, which was increased during an analysis run with a rate of 6°C/min to 170°C. After holding the temperature for 0.5 min, it was finally increased with a rate 10°C/min to 270°C and held for 10 min. Then the temperature was decreased with a rate of 20°C/min to 100°C and held for 0.5 min. As carrier gas, nitrogen was used with a flow rate of 38 ml/min. The FID was supplied with hydrogen (40ml/min) and synthetic air (400ml/min) and was run with split injection mode with a ratio of 1:25. Analysis of FID-signals was done with computer and HP 3365 series II chem-station (Version A.03.11) software.

Identification of the sample peaks was done by comparison with retention times of the methylized standard substances. The quantification was achieved by measuring the peak area of the samples in relation to the internal standard with known amount (phenyl-β-D-plucopyranoside). The following standard substances were used: glucose, fructose, sucrose, trehalose, glycerol, sorbitol, mannitol, and myo-inositol. Additionally two known concentrations of these standard substances were used as a calibration control. These substances are commonly occurring cryo-protectants in insects (Sømme, 1982; Block, 1990; Leather et al., 1993; Bale, 2002). Other, rarely occurring anti-freeze substances as threitol, erythritol, arabitol, and several others have been neglected because of technical limits and time causes.

*3.2.3.5 Experiments*

3.2.3.5.1 Experiment 1:

It was investigated if two different acclimatisation regimes, together with diapause and non-diapause and two different temperatures had an influence in the production of possible cryo-protectants compared to a control (no diapause, 21°C, long-day, one transfer to a cage in the climate chamber) and a no acclimatisation regime. All treatments were done with 10 to 15 individuals each. A summary of treatments gives table 8. In all treatments, flies were kept for one week in cold conditions (5° or 0°C, respectively). To facilitate the experiment 40-50 individuals were kept in the cold conditions to have afterwards a sufficient number of live flies present, as the mortality was high.

Acclimatisation 1: ½h 15°C and 1h 10°C.

Acclimatisation 2: 4 days at 15°C, 1 day at 10°C, 10 days at 5°C (abbreviated treatment similar to Hart & Bale, 1997b).

**Table 8:** Summary of treatments for the determination of possible cryo-protectants of *E. balteatus* in experiment 1.

| Treatment No. | Acclimatisation regime | Diapause status | Temperature (°C) |
|---|---|---|---|
| 1 | No acclimatisation (Control) | No diapause | 21 |
| 2 | No acclimatisation | No diapause | 5 |
| 3 | No acclimatisation | Diapause | 5 |
| 4 | Acclimatisation 1 | Diapause | 5 |
| 5 | Acclimatisation 2 | Diapause | 5 |
| 6 | Acclimatisation 1 | No diapause | 5 |
| 7 | Acclimatisation 2 | No diapause | 5 |
| 8 | No acclimatisation | No diapause | 0 |
| 9 | No acclimatisation | Diapause | 0 |
| 10 | Acclimatisation 1 | Diapause | 0 |
| 11 | Acclimatisation 2 | Diapause | 0 |
| 12 | Acclimatisation 1 | no diapause | 0 |
| 13 | Acclimatisation 2 | no diapause | 0 |

3.2.3.5.2 Experiment 2:

An additional protocol with changing temperatures was tested with reduced treatment numbers. Night temperatures were at 0°C and day temperatures at 5°C. Otherwise flies were kept as in treatments 8, 9, 11, and 13 of experiment 1. All other experimental arrangements were as in experiment 1.

*3.2.3.6 Statistical Analysis*

Data were analysed with SPSS 12G and SAS 9. Since most the data did not met ANOVA assumptions also after square root (+3/8) transformation, the Kruskal-Wallis test was used for overall comparisons. If significant differences were found, Bonferroni-Holm adjusted multiple comparison tests were used to compare all treatments with each other within one substance.

### 3.2.4 Yellow pan trap catches and overwintering sites

*3.2.4.1 Yellow pan traps*

Beginning with 1. November 2002 until May 2003 in several supposed overwintering habitats, yellow pan traps were placed. Collecting sites were situated in five hedges, five wood lots, and three forests (*Carpinion betuli* association (oak-hornbeam forest) (Pott, 1992)), all situated in the south of Hannover near Jeinsen (9°8´ E, 52°2´ N) and Hiddestorf (9°7´ E, 52°3´ N) (figure 5, ca. 80m over sea level). In of the two forests ("Ohlendorfer Holz" and "Bettenser Holz", see figure 5) the traps were established one week later.

The landscape is a typical loess boerde (i.e., a fertile plain) belonging to the large cultivation area of "Hildesheimer Boerde". Large parts of the boerde area are reserved for conventional high-input agriculture using the very fertile soils, which has led to a loss of spatial heterogeneity due to plot realignments (Krause, 1997). Habitats like hedges, wood patches, forests, or weed strips were removed to a large extent and the remains are

relatively isolated. However, the area used in this study belongs to the more diverse landscape parts of the boerde. Farmers here concentrate cultivation mainly on winter wheat, sugar beets, and winter barley. Nevertheless, in the last years the cultivation of several additional crops is increasing, examples are potatoes, oilseed rape, maize, and several vegetables.

In each habitat type, two traps were installed. In the forests and wood patches, one trap was situated on the edge and one in the inner part of the habitat in a clearing (minimum distance from edge 25 m), not visible from outside. For this study, yellow ("rape-coloured"), rectangular plastic pan traps manufactured by Zeneca Agro with the size 32.5 x 25 x 7 cm were used. The pans had on the shorter side a small gaze-covered hole as overflow. As trapping fluid, a mixture of tap water and ethylenglycol (10:1, vol/vol) was used. A bit of acetic acid for conserving reasons and Tween 20 as detergent was added. The traps were half-filled, on emptying the traps, the fluid was refilled, and the traps cleaned if necessary. All traps were placed on wooden platforms (ca. 1.2 m high) and were sheltered by a semi-circular formed part of wire netting with ca. 8 cm mesh width (before use, the wire netting was tested if it repels *E. balteatus* flies, which was not the case). This shelter served as protection against wind and leaf litter.

Only syrphid flies were collected weekly with forceps and transferred in 70% ethanol. Later they were frozen at –20°C. During colder periods, traps were emptied biweekly. For diapause evidence, a part of the collected *E. balteatus* females were dissected and ovaries and spermathecae examined as described in chapter 3.2.1.2. Additionally the body colour of caught individuals was compared with individuals reared at standard conditions.

On 9.12.02 in the hedges 1, 2, 5 and on 17.12.02 in hedge 2, both traps were blown away by a storm.

*3.2.4.2 Overwintering sites*

In two winters (2001/02, 2002/03), the attempt was made to find overwintering sites ("hibernacula") of *E. balteatus* in several supposed habitats. Information about possible overwintering sites was gathered from literature data (Kula, 1982; Tolsgaard & Bygebjerg, 1991; Barkemeyer, 1994; Wolff, 1996) and from F. Gilbert and A. Ssymank (both personal communication) and the following sites were investigated (the number in brackets indicates the number of investigated sites):

Crevices in walls (35), crevices in walls with ivy vegetation (20), bark with ivy vegetation (10), bark crevices without ivy (20) (both on oaks, hornbeams, pines), dead wood parts (20), sheds (4), barns (1), open attics (2), and open cellars (3). Each site was investigated once up to a height of approximately 1.7 m with torch, forceps and aspirator. The sites were located in the following places: "Ohlendorfer Holz", "Bettenser Holz", "Stamsdorfer Holz", "Jeinser Holz" (see figure 5), Hannover-Eilenriede, the village area of Hiddestorf, Hannover-City, and the IPP-area. In 2003, according to Wolff (1990) additionally two dense spruce wood plantations were investigated. They were situated in the "Bettenser Holz" and near Pattensen.

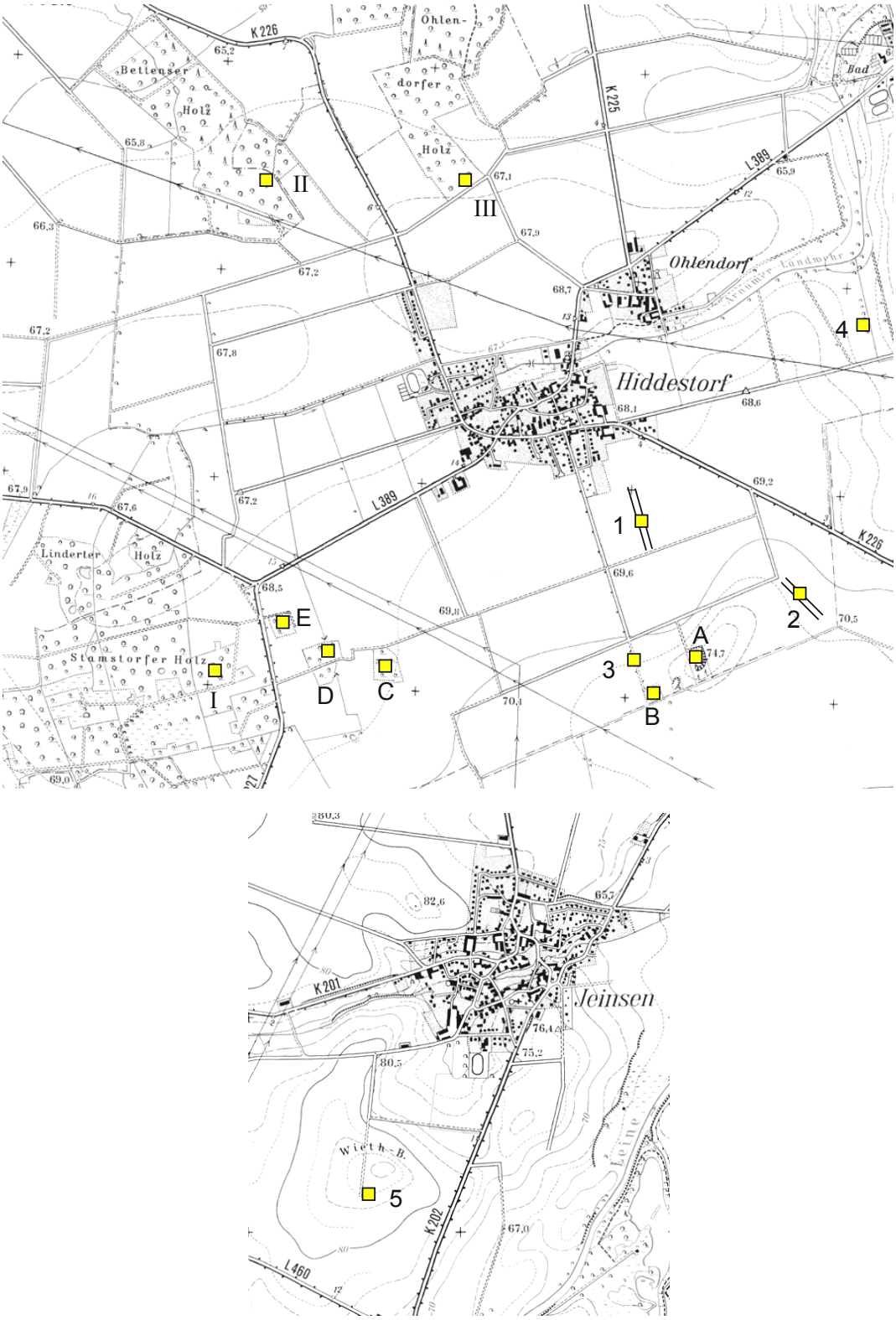


**Figure 5:** Maps of collecting sites in winter 2002/03 near Hiddestorf and near Jeinsen, both south of Hannover. Yellow dots mark yellow pan trap sites with the following numbering: 1-3= hedges, A-D= wood lots, and I-III= forests.

## 3.3 Results

3.3.1 Diapause

*3.3.1.1 Photoperiod effects on development of larvae, pupae, and adults*

Table 9 shows the developmental time of larvae and pupae reared under the five test photoperiods. A linear regression analysis failed to reveal any relationship between the time for insect development and photoperiod (larvae: $r^2 = 0.005$, $F_{1,124} = 0.6$, $P = 0.439$; pupae: $r^2 = 0.077$, $F_{1,124} = 10.2$, $P = 0.002$; both n = 25 per treatment).

**Table 9:** Mean developmental time (days) of larvae and pupae of *Episyrphus balteatus* maintained at 20°C and subjected to five different photoperiods. The times are the means from 25 insects/photoperiod.

| Photoperiod | Developmental time of larvae | Developmental time of pupae | Total |
|---|---|---|---|
| L10.5:D13.5 | 9.1 | 11 | 20.1 |
| L11.65:D12.35 | 10.5 | 7.5 | 18.0 |
| L12.5:D11.5 | 10.5 | 9.2 | 19.7 |
| L14.5:D9.5 | 9.6 | 8.5 | 18.1 |
| L15.5:D8.5 | 9.9 | 9.2 | 19.1 |
| Mean | 9.9 | 9.1 | 19.1 |
| SD | 0.59 | 1.29 | 0.98 |

In contrast, development of ovaries ceased entirely, or was reduced considerably (ovary length: $F_{5,144} = 50.4$; ovary width: $F_{5,144} = 34.6$; both $P<0.0001$), as the photoperiod was reduced (figure 6). Development of ovaries was reduced considerably at the photoperiods that corresponded to the end of September (L11.65:D12.35), mid-October (L10.5:13.5), and November (L8:D16). Such ovaries generally contained only egg

primordia and non-mature eggs. The gradual trend from short to long photoperiods was paralleled by changes from small ovaries to mature eggs (figure 6).

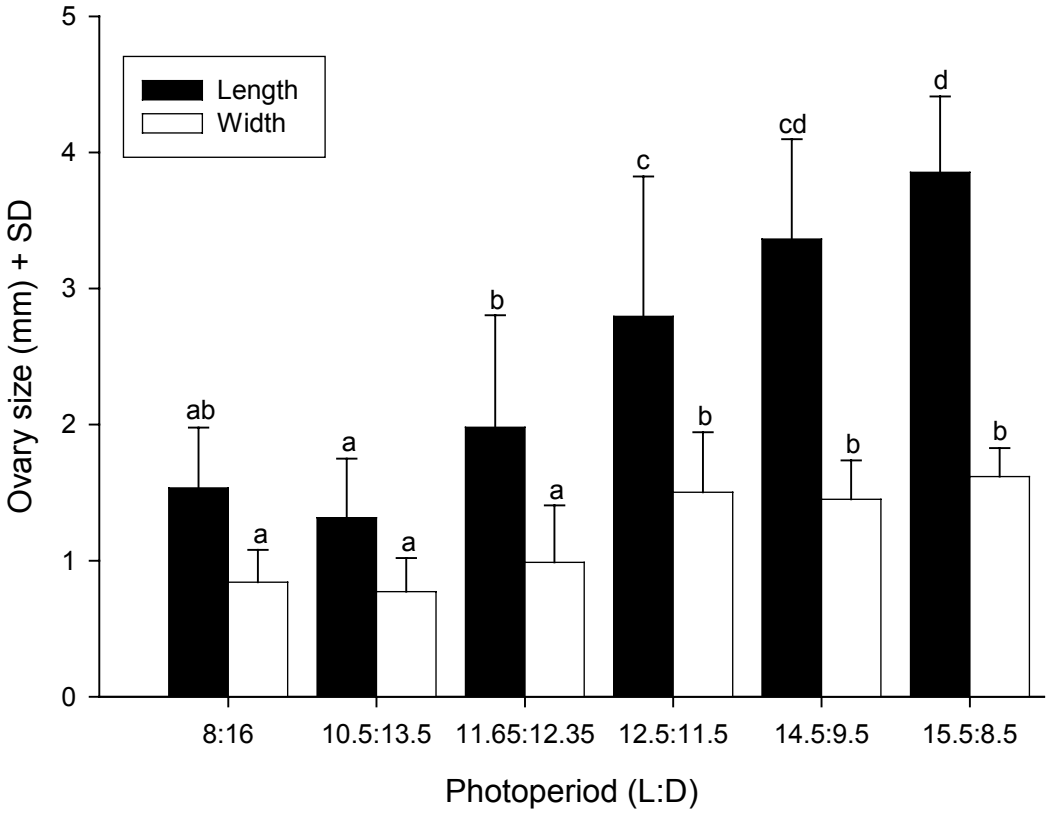


**Figure 6:** Mean sizes (+ SD) of ovaries of mated *Episyrphus balteatus* individuals reared, from the second-instar stage onwards, under six different photoperiods (n = 25 insects/photoperiod). Different letters above columns of the same shade differ at $P<0.01$.

At 20°C, the critical photoperiod at which 50% of the hoverflies entered diapause was calculated as 11.9 h using a Probit analysis, (95% fiducial limits: 10.4-13.0) ($\chi^2 = 8.3$, d.f. = 4, P = 0.081). The Gompertz distribution produced the best fit. The direct estimation from the diapause response curve (figure 7) indicated that the critical photoperiod was approximately 11.8 h. At 15°C, the critical photoperiod was calculated as 12.7 h (95%

fiducial limits: 12.2-13.1) ($\chi^2$ = 4.6, d.f. = 4, P = 0.336), with the Gompertz distribution again providing the best fit. Direct estimation (figure 7) indicated that the critical photoperiod was approximately 12.4 h.

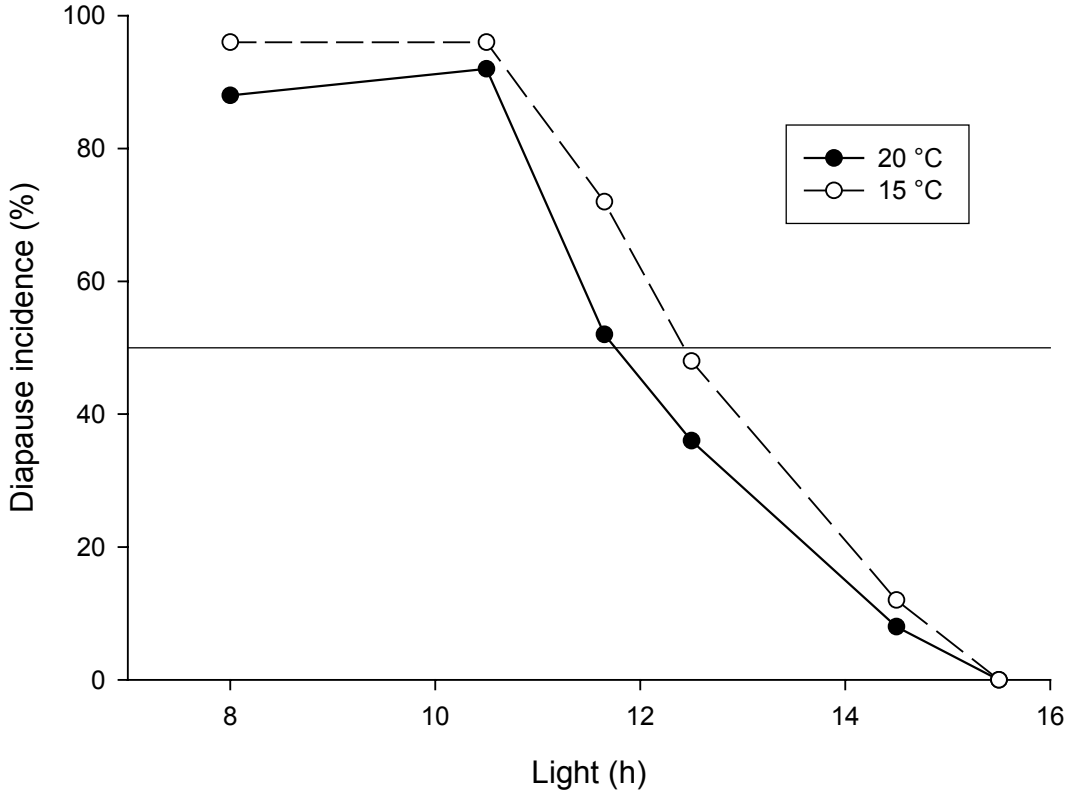


**Figure 7:** Photoperiodic response curve for female *Episyrphus balteatus* kept at 15° and 20°C. Photoperiod treatment started in $L_2$-stage. The 50% threshold is shown by the horizontal line.

*Episyrphus balteatus* adults caught in spring or autumn are often dark in colour (e.g., Holloway et al., 1997). Therefore, the colours of the insects in the various treatments were recorded to determine whether the dark colour was associated with individuals that had entered diapause. In the current tests, both diapausing and non-diapausing flies had the expected "normal" colour when reared at 20°C. The hoverflies reared at 15°C were

slightly darker than those reared at 20°C. However, there was no indication that change in colour was related to change in photoperiod.

Independent of photoperiod and temperature, all females, in which the content of the spermathecae could be distinguished clearly (85% of the total), were mated.

*3.3.1.2 Photoperiod effects on fat content in males and females*

Photoperiod had a pronounced effect on the fat content of female hoverflies ($F_{5,84}$ = 14.2, P<0.0001). As photoperiod decreased, the fat content increased, except in the hoverflies subjected to the shortest light period (8 h), in which the fat content was similar to that of the hoverflies subjected to the longest (15.5 h) photoperiod (figure 8).

In the males reared under short-photoperiod and long-photoperiod conditions, the mean (± SD) fat content was 4.98 ± 2.10% and 4.99 ± 1.55% of the fresh weight of the flies, respectively. Hence, there was no difference between the two extreme treatments (t-test: t = -0.19, P>0.05; n = 10 each), indicating that males do not accumulate energy reserves.

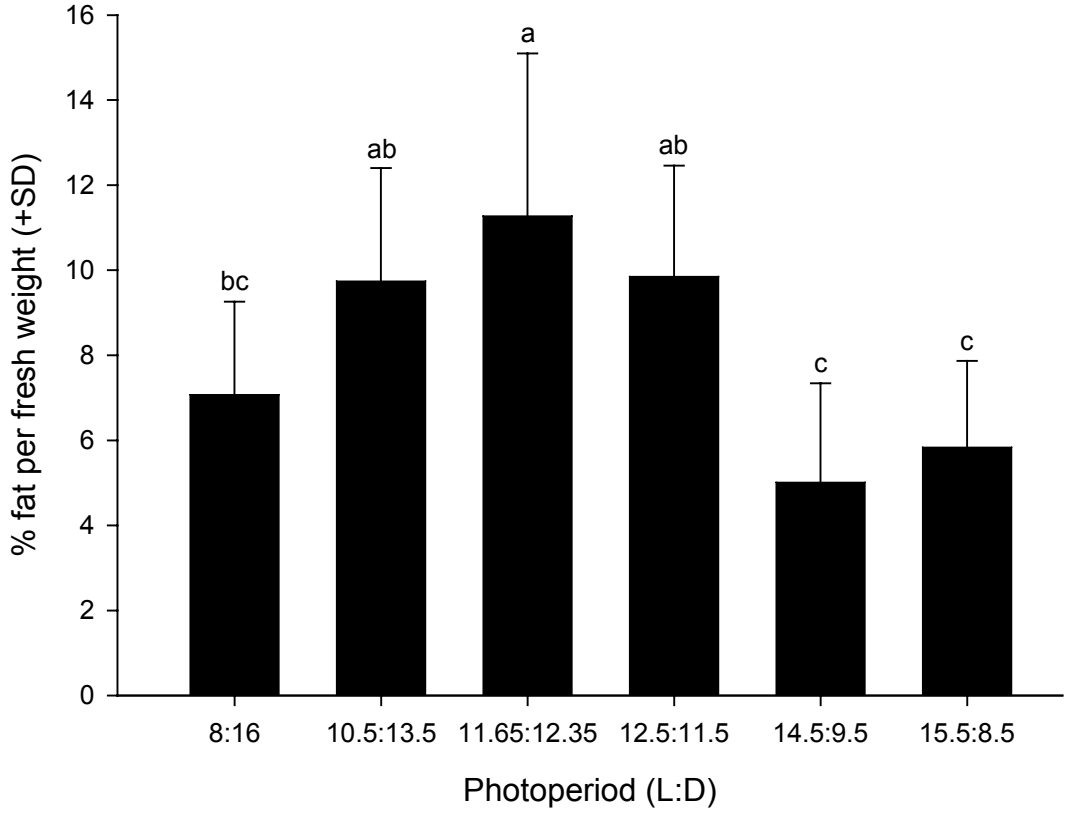


**Figure 8:** Percentage of fat per fresh weight of female *Episyrphus balteatus* reared under different photoperiods (n = 15 insects/photoperiod). Different letters indicate that values differ at $P<0.01$.

*3.3.1.3 Sensitive stages*

Exposure of the four different insect stages to a short-day photoperiod, in an attempt to induce diapause, resulted in significant differences in ovary development. Table 10 illustrates that the attempts to induce diapause by subjecting insects to a short-day treatment was effective only during the two larval stages. Adults from these two treatments had considerably reduced ovaries (ovary length: $F_{4,94} = 60.6$; ovary width: $F_{4,94} = 48.6$; both $P<0.0001$). Adults from the individuals treated at the pupal stage produced ovaries that were nearly twice as long and wide as those from the insects treated as larvae.

These ovaries were still considerably smaller than those recorded in the control treatments. In contrast, the ovaries of the treated adults were similar to those in the control treatments.

**Table 10:** Mean sizes of ovaries of *Episyrphus balteatus* females maintained at 20°C under a diapause-inducing short-day photoperiod. Individuals were transferred at different developmental stages to the short-day treatment.

| Stage at which short-day treatment started | n | Mean ovary length (mm) ± SD | Mean ovary width (mm) ± SD |
|---|---|---|---|
| $L_2$ | 20 | 1.61 ± 0.157a | 0.96 ± 0.177a |
| $L_3$ | 19 | 1.67 ± 0.694a | 0.98 ± 0.263a |
| Pupae | 20 | 3.18 ± 0.467b | 1.68 ± 0.252b |
| Teneral adult | 20 | 3.39 ± 0.699bc | 1.69 ± 0.381b |
| Control (long-day) | 20 | 3.77 ± 0.828c | 1.96 ± 0.367b |

Means followed by different letters in the same column differ at $P<0.05$ (Tukey-Kramer test).

*3.3.1.4 Diapause termination*

At 20°C, subjecting diapausing females to photoperiods longer than 10.5 h affected ovarian development (ovary length: $F_{4,96} = 247.8$; ovary width: $F_{4,96} = 101.0$; both $P<0.0001$). Only the long-day photoperiods with 12.5 h, 14.5 h, and 15.5h light were effective in terminating diapause. After 2 weeks under such conditions, the sizes of the ovaries had increased considerably and mature eggs were present (table 11). Hence, our findings indicate that for 4-week-old females, diapause should be terminated towards the end of March at Hannover, Germany. The numbers of individuals in diapause varied from

95% in the short-day (10.5 h) treatment to 5% in the long-day (15.5 h) treatment. At all tested photoperiods, more individuals remained in diapause at 15° than at 20°C (table 11).

**Table 11:** Mean sizes of ovaries of *Episyrphus balteatus* females maintained at 20°C together with the percentage of individuals that entered diapause at both 20°C and 15°C (n = 20 hoverflies/photoperiod). To assess the termination of diapause the hoverfly larvae were reared under a diapause-inducing short-day photoperiod before being transferred to the longer photoperiods.

| Photoperiod after initial short-day treatment | Mean ovary length (mm) ± SD | Mean ovary width (mm) ± SD | Percentage of diapausing individuals at 20°C | Percentage of diapausing individuals at 15°C |
|---|---|---|---|---|
| L10.5:D13.5 (control) | 1.38 ± 0.342a | 0.76 ± 0.238a | 95 | 96 |
| L11.65:D12.35 | 1.75 ± 0.684a | 0.77 ± 0.209a | 70 | 82 |
| L12.5:D11.5 | 3.21 ± 0.737b | 1.53 ± 0.270b | 40 | 55 |
| L14.5:D9.5 | 3.89 ± 0.467b | 1.63 ± 0.178b | 10 | 23 |
| L15.5:D8.5 | 3.85 ± 0.596b | 1.62 ± 0.226b | 5 | not tested |

Means in the same column with different letters differ at $P<0.05$ (Tukey's HSD test).

*3.3.1.5 Semi-field experiments*

Figure 9 provides an overview of the weather data, the survival of the adults, and the phenological data on all stages of the hoverflies. From September onwards, percent survival was calculated each week for the adults collected from the field together with those actually reared in the field cages. Individuals that could not be accounted for at the end of the experiment (see below) were not considered. The last egg was found on 26 September and some of the associated larvae survived until 7 January. However, none of these larvae developed to the adult stage. The last larva pupated on 3 October and some

pupae survived until 16 December. The last adult eclosed on 19 November. No hoverfly adults eclosed from any of the remaining pupae. The mortality of the hoverfly adults was high (females = 87%; males = 100%). The last male died on 16 December 1999, whereas surviving females were found until 15 March 2000. A multiple linear regression analysis indicated that female mortality was correlated most strongly with the duration of the experiment and with humidity. For male mortality, the strongest correlations were with rainfall and temperature. The overall mortality can only be explained partly by the factors recorded, as the correlations were not strong (table 12).

**Table 12:** Stepwise multiple regression analysis of the mortality recorded weekly for male and female *Episyrphus balteatus* kept in the semi-field experiment. Temperature was forced into the model.

| Regression equation | $r^2$ | F | d.f. | P |
|---|---|---|---|---|
| Female mortality = -0.257 - 0.001 duration -0.051 temperature + 0.175 humidity | 0.453 | 6.631 | 27 | 0.002 |
| Male mortality = 1.444 - 0.012 temperature -0.109 rainfall | 0.411 | 4.532 | 15 | 0.032 |

Both males and females rested on the walls and bottoms of the cages instead of entering the hiding places provided as potential overwintering refuges. In addition, some adults flew on both cool (up to 7°C) and warm days, and were observed foraging and drinking. The winter of 1999/2000 was mild in Hannover. The coldest temperature recorded in the field cages was –5°C and there were only 8 days during which the average temperature was below 0°C. The relative humidity in the cages was high, averaging approximately 91% (max: 100%, min: 60%) throughout the experimental period.

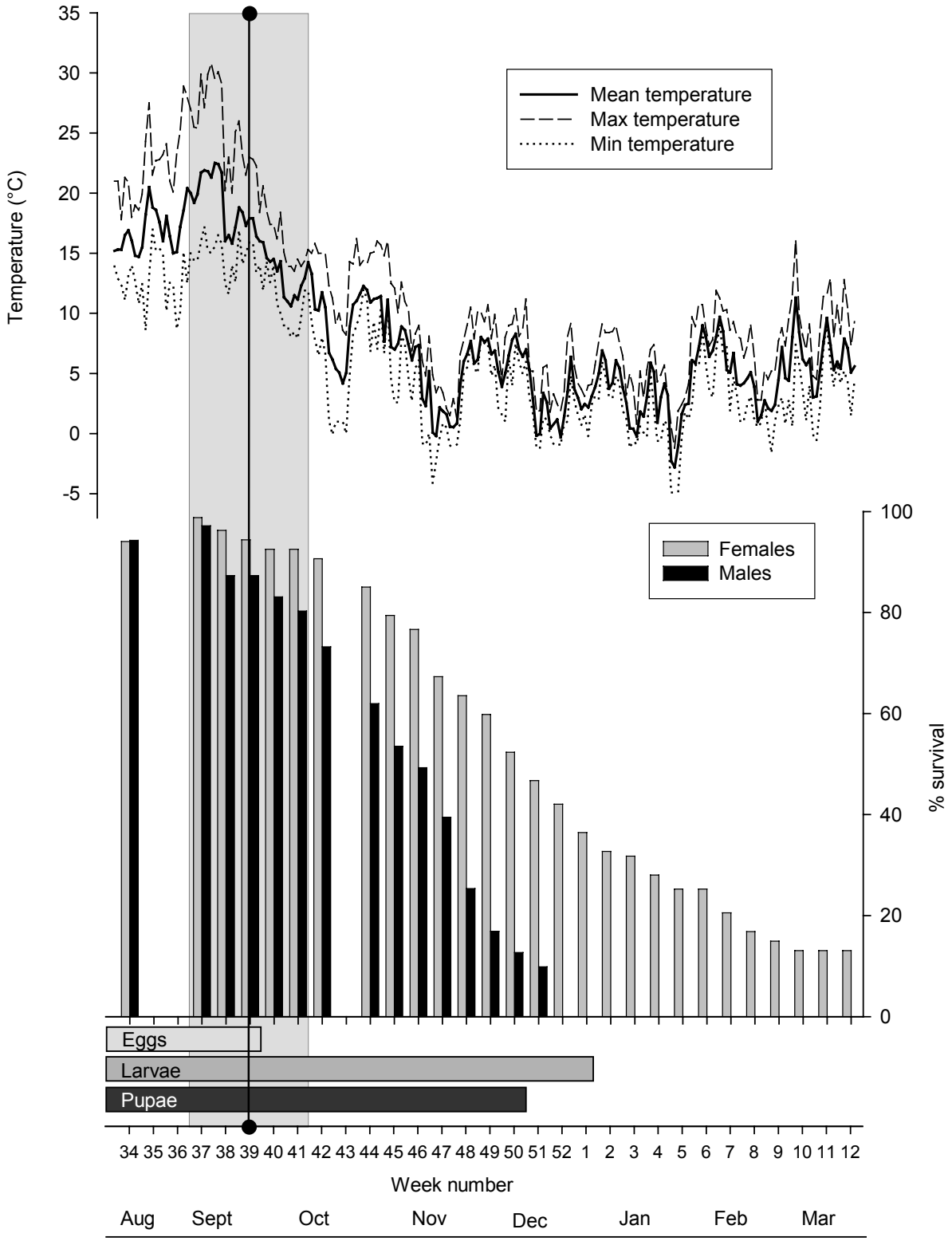


**Figure 9:** Phenology of *Episyrphus balteatus*: Developmental stages (horizontal bars), survival of male and female adults (vertical bars), and ambient temperatures (lines) recorded during the semi-field experiment. The critical photoperiod (± 95% fiducial limits as grey shade) is plotted as the vertical line.

### 3.3.2 Cold hardiness

*3.3.2.1 Lethal time determination*

In females and males, there was a strong correlation between time and mortality in all treatments: with increasing duration of the experiment, mortality increased (non-linear regressions, most $r^2$ values > 0.7, all models are fitting with significant P-values, see table 13). The Probit analysis also produced well fitting models with not significant P-values (table 14). This relation between mortality and time is more distinct in the cold treatments compared to controls with 21°C. With decreasing temperature, in all treatments the mortality was increasing and survival times became shorter (figures 10, 12, 14, 16). The acclimated-diapausing females were always the most cold hardy ones and the standard, i.e., non acclimated and not diapausing flies were the most susceptible individuals.

In the control treatments diapause had no influence on longevity compared to the non-diapausing specimen, both mortality rates increased after 250h (ca. 14 days) rapidly (figure 10). In the 5°C experiment, lifetime was not increased compared to both controls but reduced in all treatments except the diapause treatment. Nevertheless, in the standard and acclimated groups both the LT50 and the LT95 values were significantly reduced, compared with both diapause groups. Between diapause and diapause-acclimatisation treatment there was no difference in LT50 and LT95 (figure 11). In other words, only the diapause stage increased survival at 5°C. The regression curves show a linear relation only in the diapause-acclimatisation treatment, in the standard and in the acclimatisation treatment the curve is almost exponential increasing (figure 10). However, the diapause curve shows after a strong increase of mortality a saturation after ca 250 h at the 40% level, that corresponds to the highest LT95 value in this experiment.

In the 0°C experiment 100% mortality was reached in the standard treatment after less than 200h and only the combination of acclimatisation and diapause reduced mortality apparently, the slope of the mortality curve is less steep compared to the other treatments

(figure 12). Nevertheless, the LT50 and LT95 values did not differ significantly with the exception of the combination of diapause and acclimatisation. Here the values are significantly higher than in all other treatments. Hence the combination of acclimatisation and diapause seems to increase cold-hardiness at 0°C (figure 13).

In the –5°C experiment survival times were again considerably reduced compared to the control treatments, 100% mortality was reached after 120-144h (figure 14). Both acclimated and diapausing and acclimated individuals were most cold hardy ones with longest survival times, but there was no significant difference in LT50 and LT95 values between all treatments (figure 15). The regression lines show a nearly linear relation between mortality and time, which skips to a saturation curve in standard and diapause treatments (figure 14).

During -10°C, survival times were the shortest, there was only a tendency of decreasing mortality (LT50 and LT95) in acclimated females of *E. balteatus* and a significant difference to diapause and standard treatments in individuals with diapause and acclimatisation treatment (figure 16, 17). Although the 100% mortality level was reached in a similar time as in the –5°C experiment, all flies were dead after maximum 144h, suggesting that acclimatisation and diapause only retarded the lethal effect of frost. The regression between mortality and time is again nearly linear in acclimated flies and flies with combination of diapause and acclimatisation, and a saturation curve in standard and diapause treatments (figure 16).

In all treatments, males were less cold hardy than females: The lethal times were shorter and the mortality rates higher. This difference was increasing with decreasing temperatures: in the 5°C treatment, lethal times were in the same range as females, in the 0°C and –5°C survival times were considerably reduced (figure 18, 19). In the –5°C treatment after 96h all male flies were dead, in the –10°C treatment (data not shown), mortality reached 100% already after 24h in all treatments. Within the compared

treatments (temperatures), there were no significant differences in the LT50 and LT95 values (figure 19). Due to statistical reasons, it was not possible to test for differences between treatments as this were experiments in different climate chambers at different times. Therefore, the acclimatisation treatment had no significant effect in all treatments, although there is a trend to longer lethal times compared with standard treatment (figure 18).

**Table 13:** Linear regression coefficients and probabilities in male and female *E. balteatus* mortalities during different low temperatures with different pre-treatment. In males diapause experiments were omitted.

| Temperature (°C) | Treatment | females | | | | males | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $r^2$ | F | d.f. | P | $r^2$ | F | d.f. | P |
| 21 | standard | 0.692 | 12.34 | 13 | 0.0015 | 0.980 | 214.28 | 13 | <0.0001 |
| | diapause | 0.715 | 13.77 | 13 | 0.0010 | - | - | - | - |
| 5 | standard | 0.983 | 326.54 | 13 | <0.0001 | 0.980 | 272.00 | 13 | <0.0001 |
| | diapause | 0.873 | 37.68 | 13 | <0.0001 | - | - | - | - |
| | acclimated | 0.961 | 135.79 | 13 | <0.0001 | 0.923 | 65.72 | 13 | <0.0001 |
| | Diapause & acclimated | 0.945 | 93.94 | 13 | <0.0001 | - | - | - | - |
| 0 | standard | 0.981 | 209.73 | 10 | <0.0001 | 0.982 | 215.92 | 10 | <0.0001 |
| | diapause | 0.987 | 314.67 | 10 | <0.0001 | - | - | - | - |
| | acclimated | 0.995 | 728.20 | 10 | <0.0001 | 0.950 | 76.10 | 10 | <0.0001 |
| | Diapause & acclimated | 0.921 | 46.81 | 10 | <0.0001 | - | - | - | - |
| -5 | standard | 0.993 | 202.40 | 5 | 0.0006 | 0.987 | 112.55 | 5 | 0.0015 |
| | diapause | 0.968 | 44.75 | 5 | 0.0058 | - | - | - | - |
| | acclimated | 0.939 | 22.87 | 5 | 0.0153 | 0.948 | 27.35 | 5 | 0.012 |
| | Diapause & acclimated | 0.950 | 28.29 | 5 | 0.0113 | - | - | - | - |
| -10 | standard | 0.949 | 28.17 | 5 | 0.0114 | - | - | - | - |
| | diapause | 0.966 | 42.63 | 5 | 0.0063 | - | - | - | - |
| | acclimated | 0.956 | 32.49 | 5 | 0.0093 | - | - | - | - |
| | Diapause & acclimated | 0.998 | 719.90 | 5 | <0.0001 | - | - | - | - |

**Table 14:** $\chi^2$ values of the Pearson Goodness-of-Fit test for Probit analyses, degrees of freedom and probabilities of female and male mortality courses in different temperatures and treatments. Results of the Probit analysis are shown in figures 11, 13, 15, 17, and 19. In males diapause experiments were omitted, statistical treatment for –10°C experiments was not possible (see text for details).

| Temperature (°C) | Treatment | females | | | males | | |
|---|---|---|---|---|---|---|---|
| | | $\chi^2$ | d.f. | P | $\chi^2$ | d.f. | P |
| 21 | standard | 16.751 | 12 | 0.159 | 4.753 | 12 | 0.966 |
| | diapause | 12.797 | 12 | 0.384 | - | - | - |
| 5 | standard | 4.410 | 12 | 0.975 | 9.172 | 12 | 0.688 |
| | diapause | 9.042 | 12 | 0.699 | - | - | - |
| | acclimated | 6.943 | 12 | 0.861 | 11.332 | 12 | 0.501 |
| | Diapause & acclimated | 3.642 | 12 | 0.989 | - | - | - |
| 0 | standard | 4.467 | 9 | 0.878 | 2.828 | 9 | 0.971 |
| | diapause | 3.299 | 9 | 0.951 | - | - | - |
| | acclimated | 2.891 | 9 | 0.968 | 6.529 | 9 | 0.686 |
| | Diapause & acclimated | 5.164 | 9 | 0.820 | - | - | - |
| -5 | standard | 0.957 | 4 | 0.916 | 1.242 | 4 | 0.871 |
| | diapause | 2.908 | 4 | 0.573 | - | - | - |
| | acclimated | 2.622 | 4 | 0.623 | 4.403 | 4 | 0.354 |
| | Diapause & acclimated | 2.437 | 4 | 0.656 | - | - | - |
| -10 | standard | 3.974 | 4 | 0.410 | - | - | - |
| | diapause | 1.271 | 4 | 0.866 | - | - | - |
| | acclimated | 1.677 | 4 | 0.795 | - | - | - |
| | Diapause & acclimated | 2.241 | 4 | 0.692 | - | - | - |

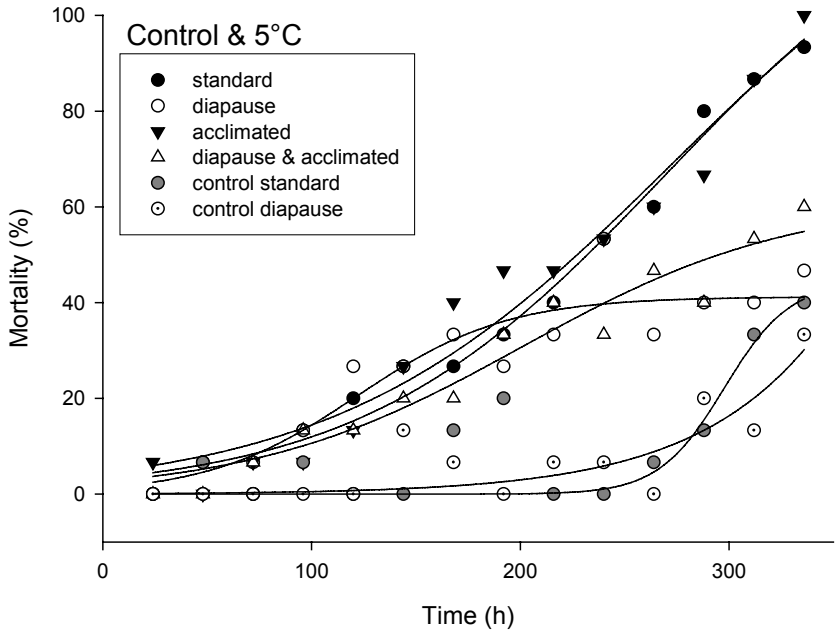


**Figure 10:** Regression curves of mortality of female *E. balteatus* during exposure to 21°C (control) and 5°C for increasing periods (lethal time). Individuals were exposed to four different treatments, see text for details.

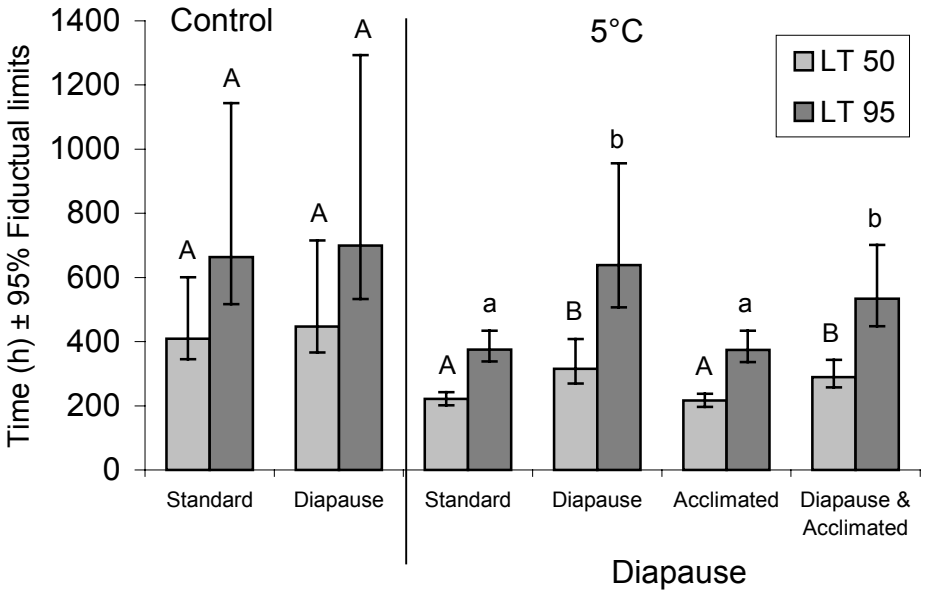


**Figure 11:** Times necessary for 50 and 95% mortality (LT 50, LT 95) in female *E. balteatus* exposed to two different temperatures and treatments. Columns of the same colour with different letters are assumed to be statistically different.

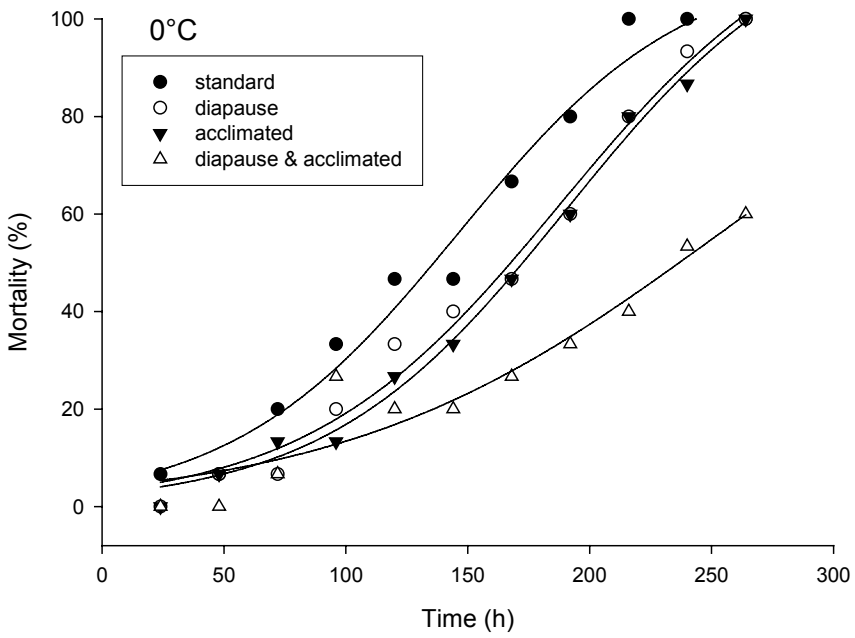


**Figure 12:** Regression curves of mortality of female *E. balteatus* during exposure to 0°C for increasing periods (lethal time). Individuals were exposed to four different treatments, see text for details.

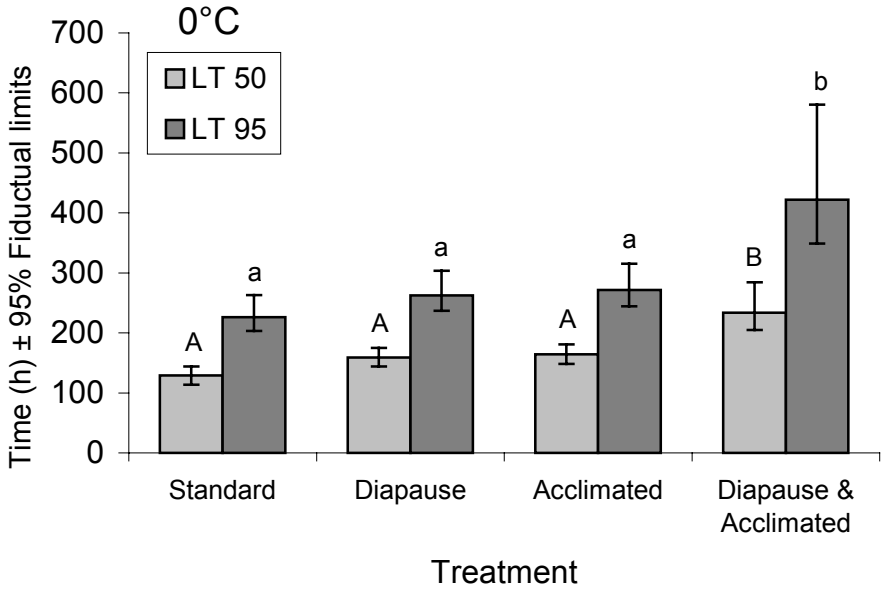


**Figure 13:** Times necessary for 50 and 95% mortality (LT 50, LT 95) in female *E. balteatus* exposed to 0°C and different treatments. Columns of the same colour with different letters are assumed to be statistically different.

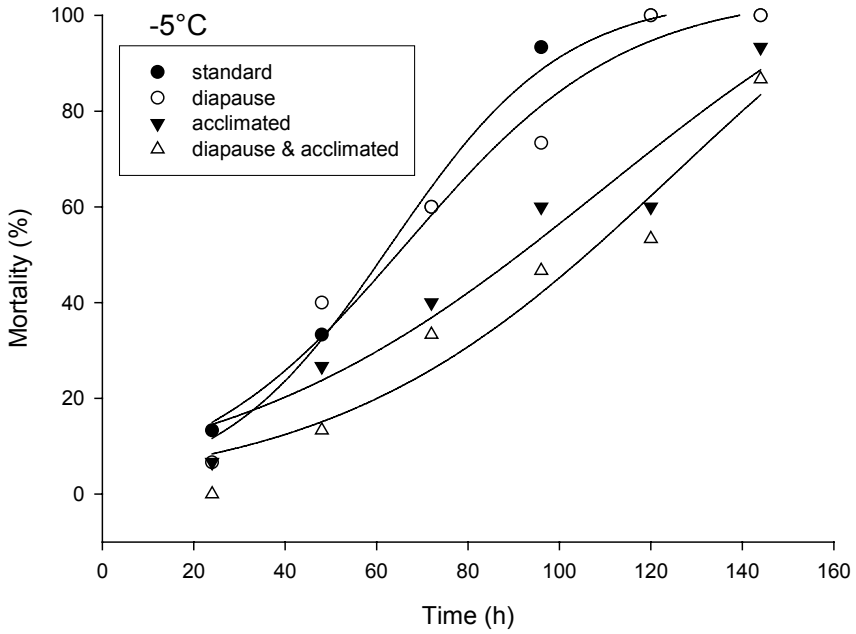


**Figure 14:** Regression curves of mortality of female *E. balteatus* during exposure to -5°C for increasing periods (lethal time). Individuals were exposed to four different treatments, see text for details.

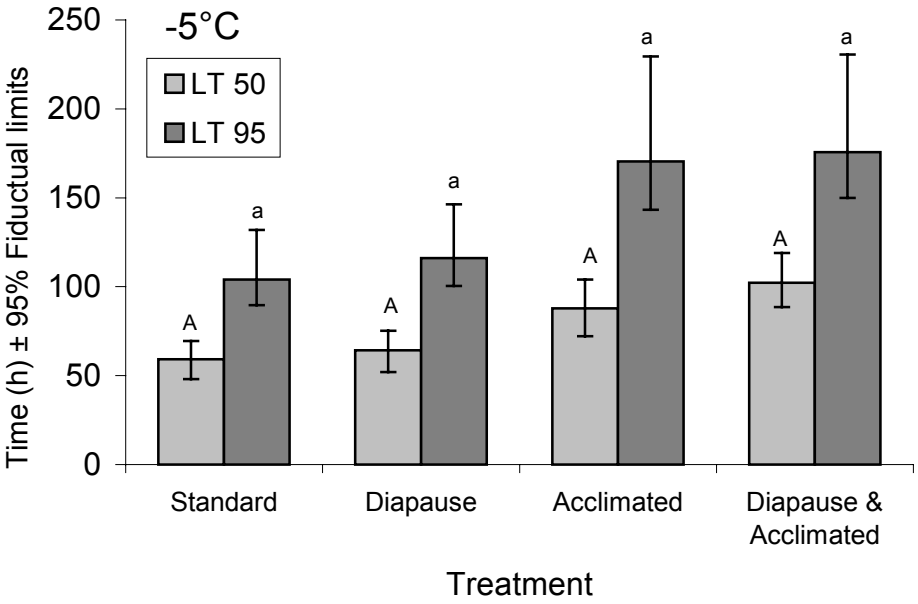


**Figure 15:** Times necessary for 50 and 95% mortality (LT 50, LT 95) in female *E. balteatus* exposed to –5°C and different treatments. Columns of the same colour with different letters are assumed to be statistically different.

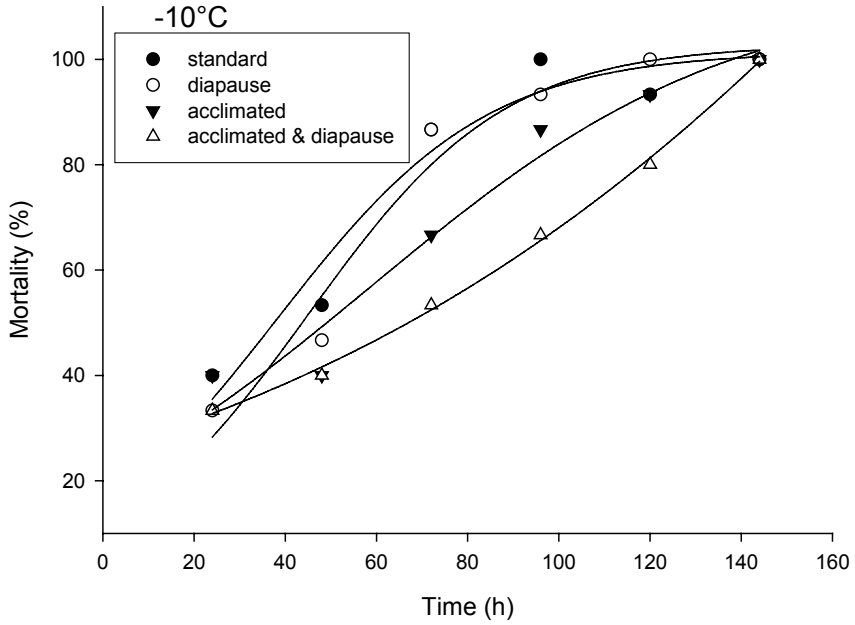


**Figure 16:** Regression curves of mortality of female *E. balteatus* during exposure to -10°C for increasing periods (lethal time). Individuals were exposed to four different treatments, see text for details.

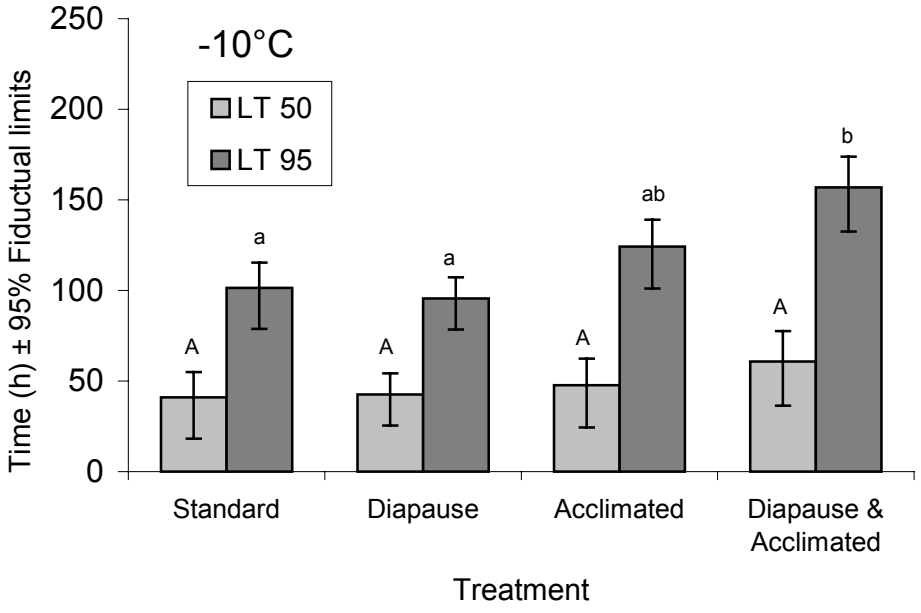


**Figure 17:** Times necessary for 50 and 95% mortality (LT 50, LT 95) in female *E. balteatus* exposed to –10°C and different treatments. Columns of the same colour with different letters are assumed to be statistically different.

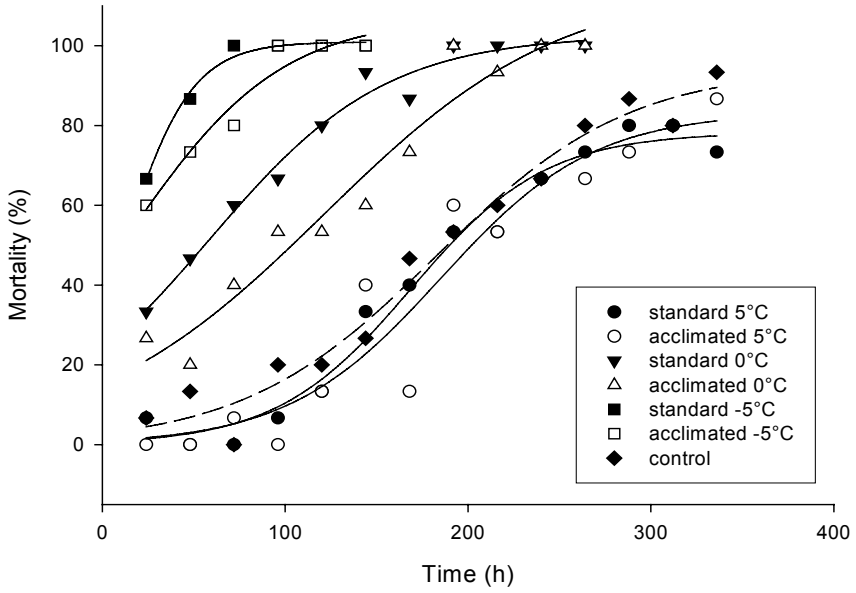


**Figure 18:** Regression lines of mortality of male *E. balteatus* during exposure to different temperatures for increasing periods (lethal time). Males were exposed to different treatments and temperatures (see text for details).

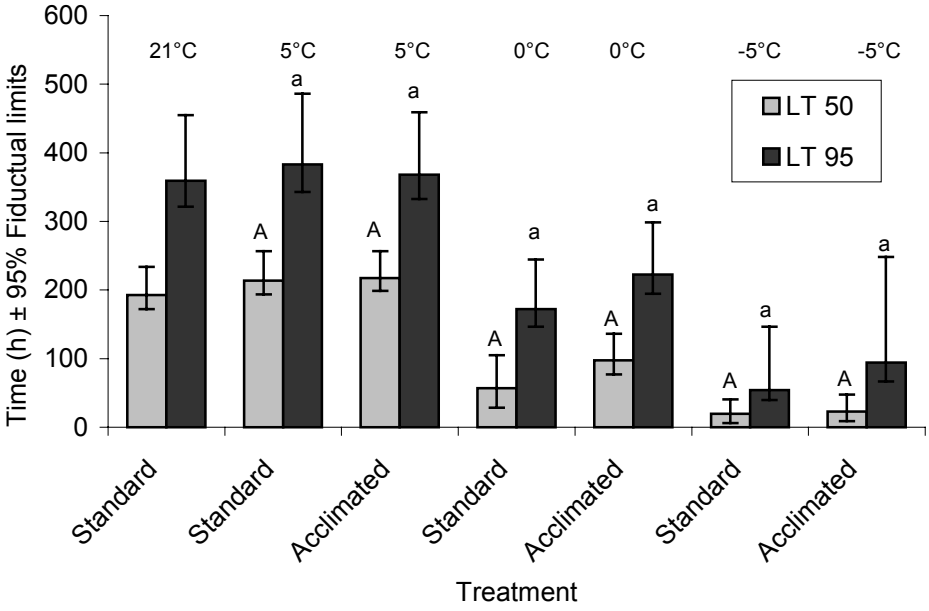


**Figure 19:** Times necessary for 50 and 95% mortality (LT50, LT95) in male *E. balteatus* exposed to different temperatures and treatments. Columns with different letters are assumed to be statistically different within one temperature.

*3.3.2.2 Reproduction after cold treatment*

Figure 20 shows the number of laid eggs 48h after the experiment. Females were exposed to 6 different cold durations and 4 treatments as described in chapter 3.2.2. On average females laid 14 eggs per day. There were no significant differences in egg numbers between cold durations (within a temperature regime) and between temperatures (within various cold durations) (table 15, 16; all $P > 0.05$). All tested females (altogether 35) were mated. As a result, cold treatments seem to have no influence on the fecundity of female *E. balteatus*.

**Table 15:** $\chi^2$ values of the $\chi^2$-test for independence for the numbers of eggs between cold durations (within a temperature), degrees of freedom and probabilities. The eggs were laid 48h after the experiment by cold-treated *E. balteatus* females.

| Treatment (Temperature, °C) | $\chi^2$ | d.f. | P |
|---|---|---|---|
| 5 | 2.93 | 5 | 0.712 |
| 0 | 3.65 | 4 | 0.455 |
| -5 | 0.16 | 1 | 0.689 |
| 21 (control) | 6.92 | 5 | 0.227 |

**Table 16:** $\chi^2$ values of the $\chi^2$-test for independence of the numbers of eggs between temperatures (within cold duration), degrees of freedom and probabilities. The eggs were laid 48h after the experiment by cold-treated *E. balteatus* females.

| Duration of cold treatment (h) | $\chi^2$ | d.f. | P |
|---|---|---|---|
| 48 | 4.78 | 3 | 0.189 |
| 72 | 2.12 | 3 | 0.548 |
| 120 | 0.35 | 2 | 0.839 |
| 144 | 0.47 | 2 | 0.791 |
| 168 | 1.05 | 2 | 0.590 |
| 240 | 1.10 | 1 | 0.300 |

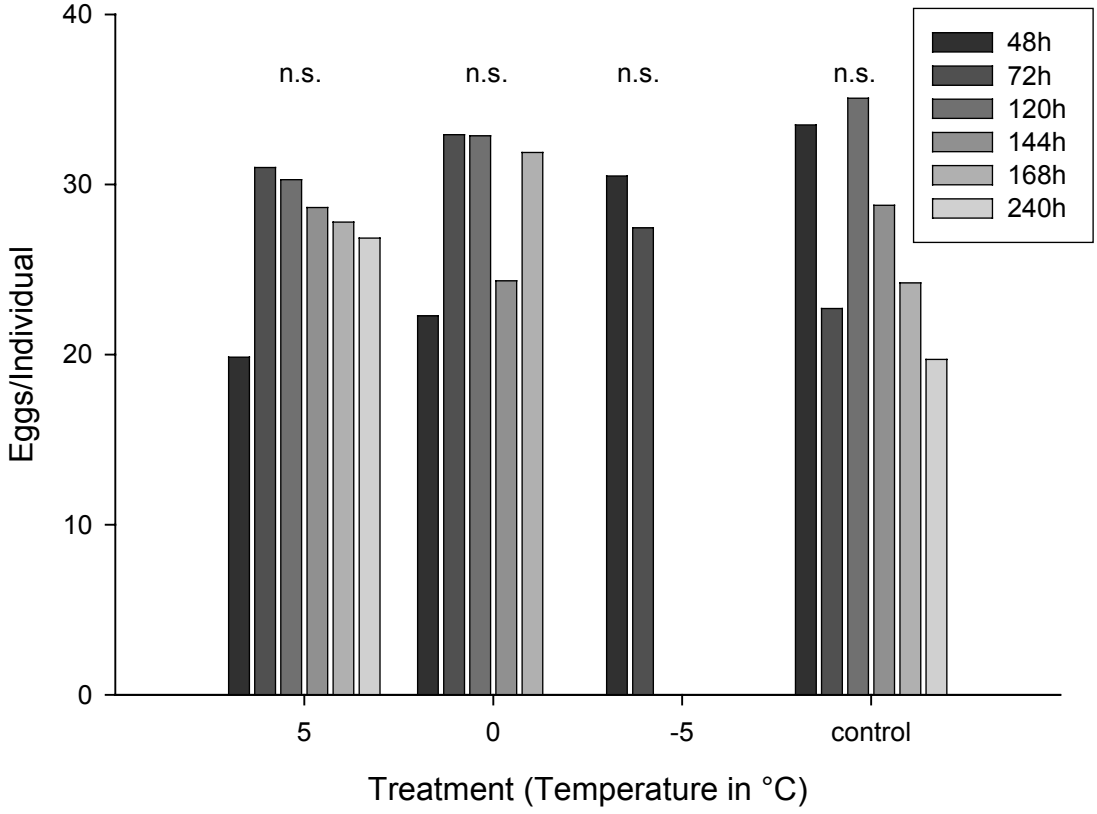


**Figure 20:** Egg number per female *E. balteatus* 48h after end of the cold treatment. Only females from six time intervals (i.e., duration of cold treatment) were investigated.

### 3.3.3 Cryo-protectant analysis

Table 17 shows the results of the first experiment of the gas chromatographic analysis of possible cryo-protectants in *E. balteatus*, table 19 of the second experiment. In both experiments, except mannitol, all substances were detectable, fructose and glucose with highest concentrations, glycerol and sorbitol with lower concentrations. Trehalose and sucrose were only in very low concentrations detectable. Myo-inosit was detectable in traces, which may be come from errors in peak area measurement.

All means had very high standard deviations resulting from high differences in sugar and polyol content between individuals (table 17, 19). Altogether only little differences were found (table 18), and there were no significant increase in the content of the polyols and sugars detectable that was induced by the treatments (i.e. constant and/or variable cold temperatures in combination with diapause and/or acclimatisation treatments) (table 17, 18 19; figure 21, 22). The only significant effect was a decrease in fructose and glucose content in *E. balteatus* compared with the control treatment in both experiments, for example in treatments numbers 3-5, 8, 10, 11-13 (figure 21, 22). This decrease occurred in experiment 1 especially in the 0°C treatments (10-13) except treatment 9, independent from acclimatisation type or diapause status, in experiment 2 in all treatments, except treatment number 2. Additionally there was a slight but not significant increase in sorbitol content in treatments 5, 8, 10, and 11 compared with the control treatment.

**Table 17:** Experiment 1: Mean content of sugars and polyols (µg/mg fresh weight ± standard deviation) in *E. balteatus* females after six different treatments at constant 5°C (treatment 2-7) or 0°C (treatment 8-13). Treatment 1 was the overall control. Different letters indicate significant differences in the content of one substance (row-wise). Post-hoc significance tests were only done if overall differences were significant as indicated in table 18.

| Substance/ Treatment* | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| glycerol | 0.493 ± 0.375 | 0.612 ± 0.984 | 0.502 ± 0.440 | 0.671 ± 0.694 | 0.655 ± 0.332 | 0.679 ± 0.671 | 0.429 ± 0.269 | 0.444 ± 0.331 | 0.684 ± 0.306 | 0.735 ± 0.607 | 0.779 ± 0.621 | 0.661 ± 0.377 | 0.552 ± 0.278 |
| fructose | 2.734 ± 0.977a | 1.162 ± 0.964bc | 2.325 ± 0.554ac | 1.413 ± 0.453ab | 1.419 ± 0.872ab | 1.625 ± 0.667ab | 1.196 ± 0.579b | 1.106 ± 0.366b | 1.445 ± 0.32ab | 1.095 ± 0.458b | 0.923 ± 0.398b | 1.035 ± 0.258b | 1.093 ± 0.332b |
| glucose | 2.002 ± 1.172c | 0.804 ± 0.505ab | 0.936 ± 0.311ab | 1.316 ± 0.483ac | 0.765 ± 0.286ab | 0.606 ± 0.385b | 1.191 ± 0.823abc | 1.032 ± 0.259a | 1.083 ± 0.373ac | 0.509 ± 0.289b | 0.835 ± 0.326ab | 0.739 ± 0.378ab | 0.827 ± 0.254ab |
| trehalose | 0.009 ± 0.015ab | 0.009 ± 0.011ab | 0.003 ± 0.007a | 0.009 ± 0.013ab | 0.010 ± 0.009ab | 0.057 ± 0.159ab | 0.003 ± 0.007a | 0.023 ± 0.016b | 0.005 ± 0.008ac | 0.013 ± 0.008bc | 0.016 ± 0.014bc | 0.002 ± 0.004a | 0.003 ± 0.007a |
| sucrose | 0.046 ± 0.032ab | 0.050 ± 0.022a | 0.017 ± 0.014b | 0.021 ± 0.014b | 0.098 ± 0.090ac | 0.120 ± 0.272ab | 0.027 ± 0.026ab | 0.020 ± 0.017b | 0.018 ± 0.015b | 0.042 ± 0.036ab | 0.071 ± 0.031ac | 0.044 ± 0.034ab | 0.029 ± 0.026ab |
| mannitol | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.001 ± 0.003 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| sorbitol | 0.546 ± 0.442 | 0.463 ± 0.280 | 0.606 ± 0.486 | 0.555 ± 0.280 | 0.631 ± 0.332 | 0.263 ± 0.351 | 0.564 ± 0.290 | 0.646 ± 0.413 | 0.475 ± 0.278 | 0.680 ± 0.449 | 0.801 ± 0.476 | 0.523 ± 0.353 | 0.448 ± 0.227 |
| myo-inosit | 0.001 ± 0.003 | 0.003 ± 0.007 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.001 ± 0.003 | 0.003 ± 0.007 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.001 ± 0.003 | 0.00 ± 0.00 | 0.003 ± 0.005 | 0.00 ± 0.00 | 0.001 ± 0.003 |

*See table 8 for description of treatment numbers.

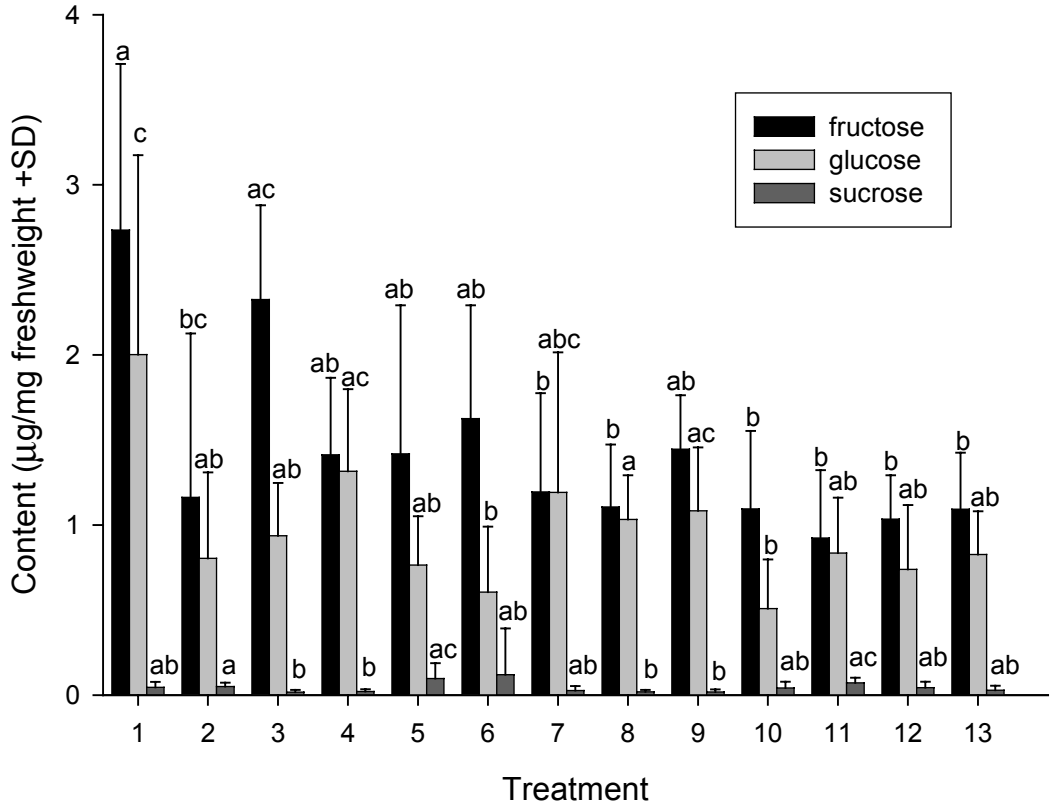


**Figure 21:** Content of most important sugars of *E. balteatus* in experiment 1. Different letters indicate significant differences between treatments within each substance (see also table 18).

**Table 18:** Overall P-values and chi-square values for possible cryo-protectants in all treatments (Kruskal-Wallis test, $\alpha$= 0.05, d.f.: 12, each for experiment 1, and 4, each for experiment 2). * indicates overall significance.

| | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|
| Substance | chi-square | P | chi-square | P |
| glycerol | 10.991 | 0.530 | 3.691 | 0.449 |
| fructose | 44.855 | >0.0001* | 26.996 | >0.0001* |
| glucose | 44.345 | >0.0001* | 18.681 | 0.001* |
| trehalose | 34.101 | 0.001* | 5.823 | 0.213 |
| sucrose | 34.713 | 0.001* | 8.459 | 0.0076* |
| mannitol | 11.500 | 0.487 | 0.000 | 1.000 |
| sorbitol | 15.642 | 0.208 | 1.738 | 0.784 |
| myo-inosit | 12.764 | 0.386 | 4.397 | 0.355 |

**Table 19:** Experiment 2: Mean content of sugars and polyols (µg/mg fresh weight ± standard deviation) in *E. balteatus* females after four different treatments at changing temperatures (1= overall control as in experiment 1; 2= no acclimatisation, no diapause; 3= diapause only; 4= acclimatisation 2 and diapause; 5= acclimatisation 2 only).

| Substance/ Treatment | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| glycerol | 0.493 ± 0.375 | 0.446 ± 0.231 | 0.577 ± 0.332 | 0.547 ± 0.283 | 0.655 ± 0.331 |
| fructose | 2.734 ± 0.977 | 1.616 ± 0.384 | 1.840 ± 0.404 | 1.07 ± 0.366 | 1.023 ± 0.402 |
| glucose | 2.002 ± 1.172 | 1.450 ± 0.424 | 1.029 ± 0.378 | 0.953 ± 0.282 | 1.109 ± 0.417 |
| trehalose | 0.009 ± 0.015 | 0.007 ± 0.013 | 0.006 ± 0.007 | 0.006 ± 0.008 | 0.013 ± 0.008 |
| sucrose | 0.046 ± 0.032 | 0.023 ± 0.018 | 0.031 ± 0.027 | 0.06 ± 0.032 | 0.036 ± 0.037 |
| mannitol | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| sorbitol | 0.546 ± 0.442 | 0.629 ± 0.238 | 0.575 ± 0.270 | 0.64 ± 0.313 | 0.601 ± 0.259 |
| myo-inosit | 0.001 ± 0.003 | 0.002 ± 0.004 | 0.00 ± 0.00 | 0.003 ± 0.007 | 0.00 ± 0.00 |

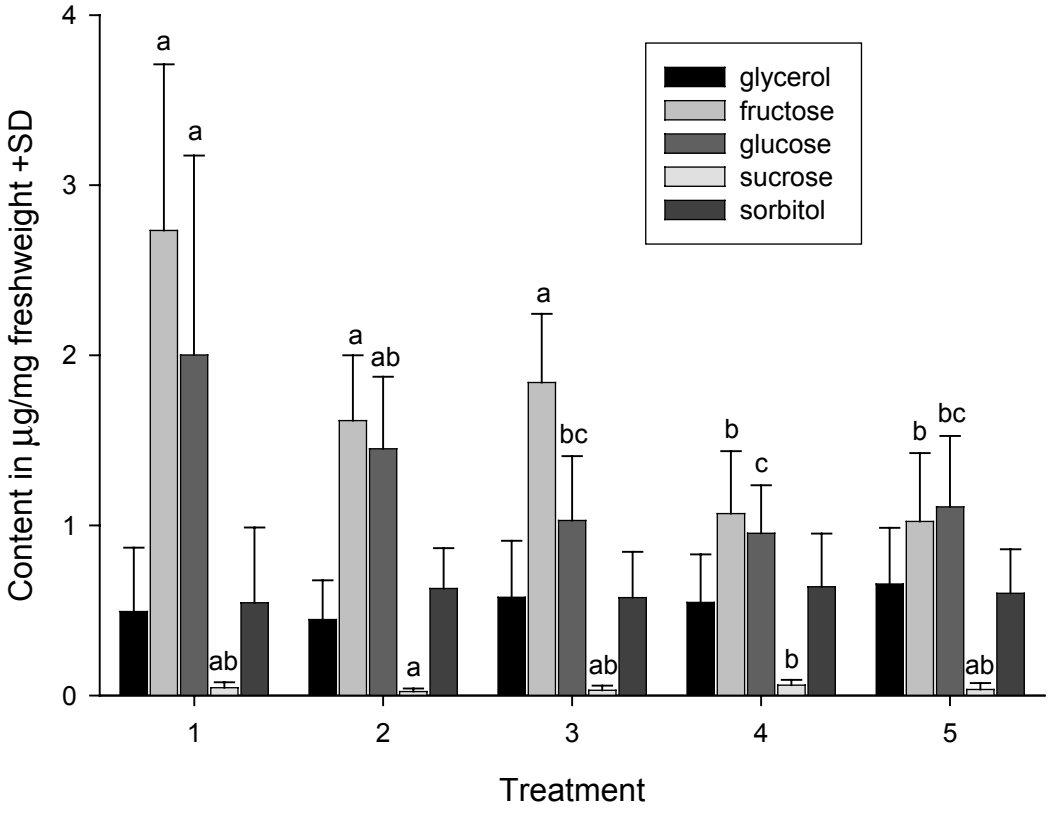


**Figure 22:** Content of most important possible cryo-protectants of *E. balteatus* females in experiment 2. Different letters indicate significant differences between treatments within each substance. In glycerol and sorbitol no significant differences were found (see table 18).

### 3.3.4 Yellow pan trap catches and overwintering sites

*3.3.4.1 Yellow pan traps*

*E. balteatus* showed during the whole winter low but continuous flight activity and the number of caught flies is not decreasing over time. The high standard deviations indicate strong differences within the habitat types (e.g. in several habitats no catches were recorded). The number of males over the complete collecting time is significantly lower than the female numbers ($\chi^2$= 12.857; P= 0.0003; d.f.= 1) and on 12.01.2003, no male flies were found at all. Surprisingly, after 1.03.2003 no syrphids were caught, although temperatures were rising (figure 24). Below 3-4°C, catches were considerable reduced and there is a good accordance between temperatures and flight activity (figure 24). No significant differences between habitat types (hedge, wood patch, forest) in total ($\chi^2$= 3.11, d.f.= 2, P= 0.211) and interior and edge of the habitats ($\chi^2$ = 0.03, d.f.= 1, P= 0.853 for wood patches and $\chi^2$= 0.06, d.f.= 1; P= 0.808 for forests) were found.

The other species caught were *Eristalis tenax* (most catches)*, Eupeodes corollae,* and *Meliscaeva auricollis*. The numbers were considerable lower than those of *E. balteatus* (figure 23).

Compared to individuals reared under long day conditions (L:D 15.5:8.5 and 21°C, data from control treatment of diapause experiments, see chapter 3.2.2.1), females out of pan traps had significantly smaller ovaries with a lack of mature eggs in all individuals. The examined laboratory-reared females had normally developed ovaries and mature eggs (table 21). In 56% of the caught females, sperm was detectable in the spermathecae. This proportion is lower compared to diapausing females from experimental rearings (see chapter 3.2.1.2) and can likely attributed to degradation in the trapping fluid, since flies were up to fourteen days in the traps. Additionally, there is a significantly relationship between body colour (table 20) and origin (trap or culture) ($\chi^2$ = 9.03, d.f.= 1, P= 0.0027),

i.e., dark body colour is related with individuals from trap catches. The fat body was only observed by view (the lipid content was not determined) and appeared to be larger than in females reared under standard conditions.

**Table 20:** Abdominal colour of winter-caught *E. balteatus* compared with reared individuals at 20°C, L:D 15.5:8.5. n= 20 and 13.

| Colour | n (caught) | n (reared) |
|---|---|---|
| "normal" | 8 | 12 |
| "dark" | 12 | 1 |

**Table 21:** Ovary size of winter-caught *E. balteatus* compared with reared individuals at 20°C, L:D 15.5:8.5. n=20 (pan trap specimen) and 25 (control specimen). Different letters indicate significant differences between rows (Mann-Whitney-U-test).

| | Caught *E. balteatus* | Reared *E. balteatus* | P |
|---|---|---|---|
| Ovary length (mean ± SD) | 1.12 ± 0.39 a | 3.78 ± 0.57 b | <0.0001 |
| Ovary width (mean ± SD) | 0.45 ± 0.10 a | 1.62 ± 0.21 b | <0.0001 |

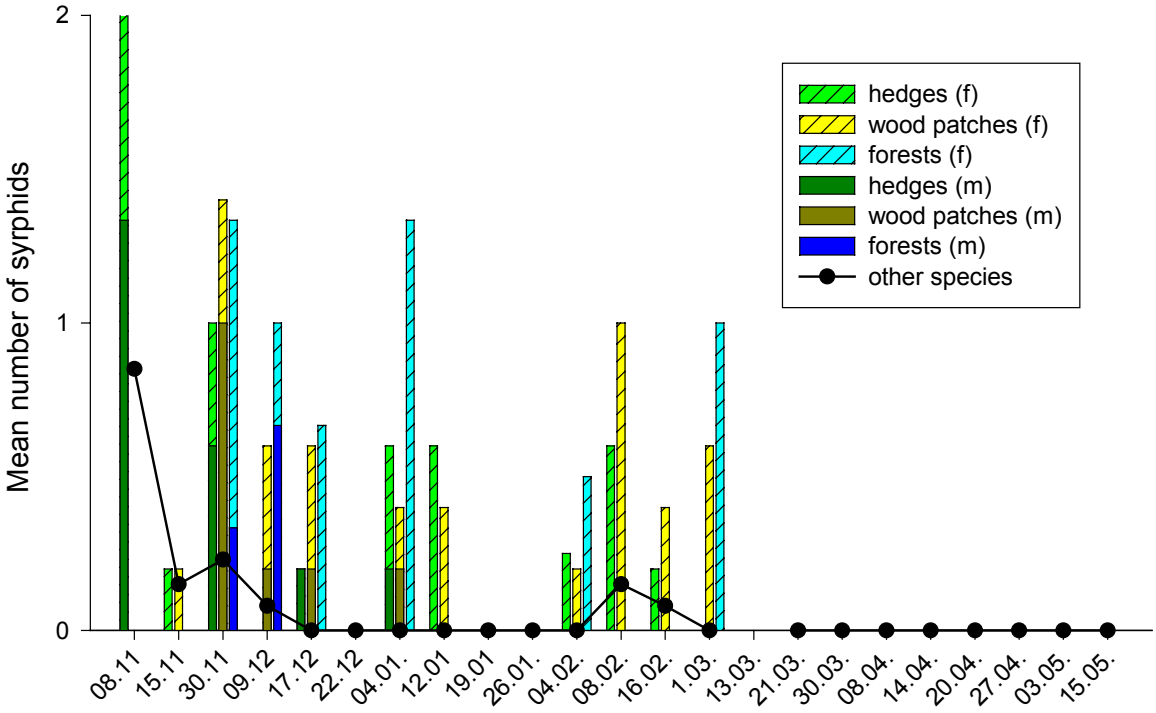


**Figure 23:** Phenology of *Episyrphus balteatus* adults (columns) contrasted by sex (f= females, m= males) and other syrphid species (line plot) caught with yellow pan traps during winter in three different habitat types.

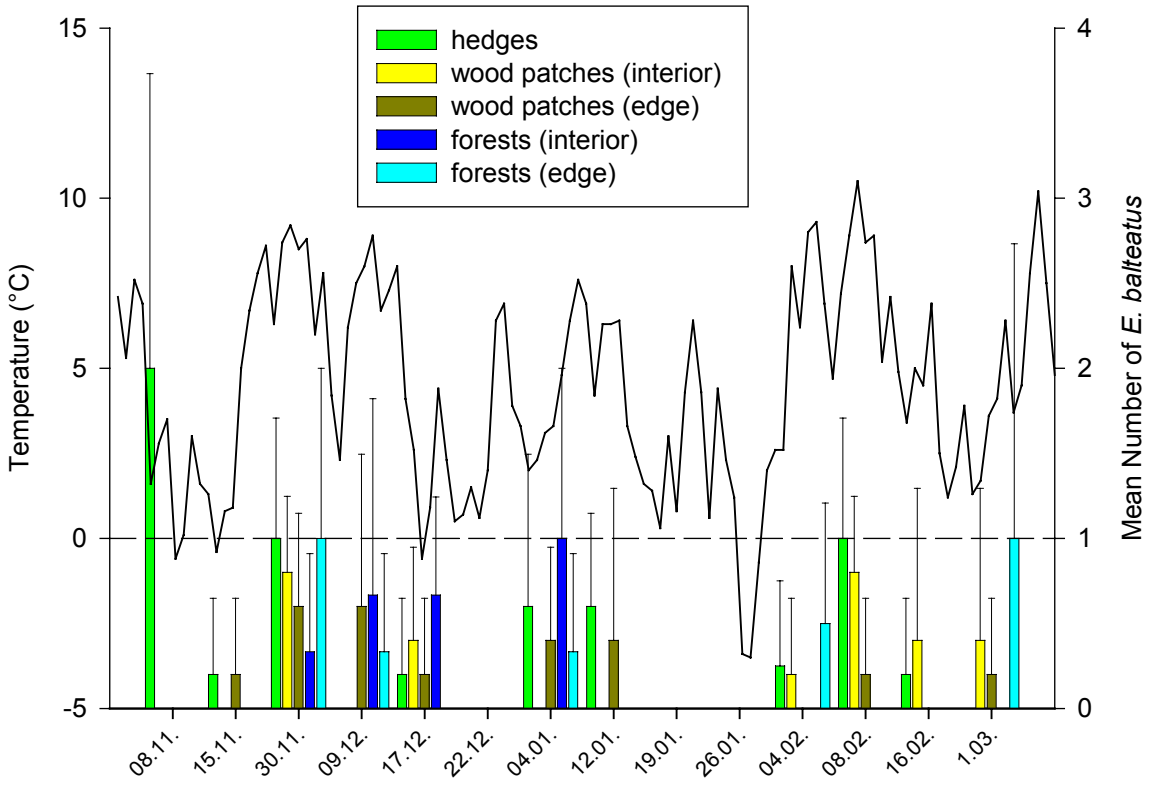


**Figure 24:** Yellow pan trap catches of *E. balteatus* (mean + SD) in different habitats (columns) and mean temperature in °C (line). Catches in wood patches and forests are separated by inner part and edge (see chapter for details). For a clearer view, dates without catches later than 1.03.2003 were left out.

### *3.3.4.2 Overwintering sites*

No flies were collected in all investigations.

## 3.4 Discussion

### 3.4.1 Diapause

The results of the laboratory experiments strongly suggest that reproductive diapause in females of *Episyrphus balteatus* is facultative, which, according to the classification of Beck (1980), is a common Type I diapause. This type of diapause is controlled predominantly by photoperiod. However, temperature can have a modifying effect, as at low temperature individuals may enter diapause at longer daylengths than they would at higher temperatures. The laboratory results are corroborated by the analysis of the females of the yellow pan trap catches during winter. These feral flies also showed significantly reduced ovary development compared to females reared under normal conditions in the laboratory and had enlarged fat bodies.

These findings support the early studies of Schneider (1948, 1969), who outlined an inhibition of ovary development and the accumulation of fat in adult *E. balteatus* during the overwintering period. Our studies also support the hypothesis that, unlike females, the males of *E. balteatus* do not overwinter in temperate regions, as they seem unable to increase the size of their fat bodies. The findings are supported by the data from the semi-field experiment, in which all males died before the end of December. In earlier studies, males were considered not to overwinter in temperate regions, as they were never observed in the early spring in such regions (Schneider, 1948; Salveter, 1996; Krause, 1997).

The lack of a correlation between photoperiod and developmental time in the larvae and pupae of *E. balteatus* indicates that photoperiod does not influence the overall time required for development. The protracted development in the semi-field experiment resulted not from short-day photoperiods, but from low temperatures. Honěk & Kocourek (1988) calculated that the lower development threshold temperature for the pupae of

*E. balteatus* was 7.5°C. Similarly, Hart et al. (1997) concluded that the low developmental threshold temperatures for all stages of this hoverfly ranged from 5° to 8°C. Hence, in relatively mild winters, some stages could survive until December or even January, and still be unable to complete their development. It is likely that the long survival of these immature hoverfly stages in this study was mainly due to the protection afforded by the cages and particularly by the artificial food supply. As results from Dušek & Láska (1974) suggested, under natural conditions most of these instars would have died much earlier. This seems in contradiction to Sarthou et al. (2005), who concluded from their results also overwintering of preimaginal stages (larvae or pupae) for south France. The only other observations about preimaginal overwintering in *E. balteatus* were reported from Dunn (1949, in Barkemeyer, 1994) who reported about overwintering pupae and different larval stages in the north of England. Additionally, Gaumont (1929, in Barkemeyer, 1994) reported from France (possibly central France), and Scott (1939) from UK (presumably near London) about overwintering larvae. Schneider (1948) stated that Gaumont (1929) also claimed to have found larval diapause in *E. balteatus*, what he questioned, as he could not replicate the results experimentally. However, the overwintering of different stages in one species is possible even in cold regions (Danks, 1978; Leather et al., 1993) and there is in general no particularly cold-hardy overwintering stage, although at a first view, the egg stage could be considered as most cold hardy. For example, the butterfly *Pararge aegeria* (Lepidoptera: Satyridae) hibernates as adult, larva, and pupa; the carabid *Pterostichus breviscornis* overwinters in all stages except the egg. In most insect orders including Diptera, all stages are found as overwintering stages and if a predominance of a particular stage is found, it is related to the life style (e.g., parasitism) and often linked to the familial strategy (Danks, 1978; Leather et al., 1993). Although the quality of the observations in the older papers cannot be estimated, the sum of the reports may indicate

that perhaps in milder climates (south France, UK) also other stages of *E. balteatus* can overwinter.

In Syrphidae, adult overwintering is not common, but in several species the overwintering stage is still unknown. Until know only following species are known as adult overwinterers: *Eristalis tenax* and surely several other species of this genus, *Eristalinus (Lathyrophthalmus) aeneus* (Scopoli), *Scaeva selenitica* (Meigen), *Scaeva pyrastri* (L.) (and surely several other species of this genus), *Eupeodes lapponicus* (Zett.), *Eupeodes luniger* (Meigen) (*E. corollae* (F.) is also assumed) and most interestingly, a close relative of *E. balteatus*: *Meliscaeva auricollis* (Meigen) (Schneider, 1958; Gauss, 1961; Brugge 1980; Hastings, 1988; Palmer, 1988; Röder, 1990; Wolff, 1990; Barkemeyer, 1994). There are no general traits (e.g., larval foraging, size, life cycle type, ecological groups, or migration status) common to all species, but no phytophagous species is present (*Eristalis* and *Eristalinus* are micro-saprophagous, all others are zoophagous). Except *E. aeneus* all species are assumed to be seasonal migrants according to Gatter & Schmid (1990).

It was calculated that at temperatures of 20° and 15°C, the critical photoperiod would occur by the end of September and towards the middle of September, respectively, in the Hannover region of Germany. As *E. balteatus* can develop from egg to adult in 21 days at 20°C but needs about 43 days at 15°C (Ankersmit et al., 1986), then the first adults could enter diapause between mid-October (20°C) and the beginning of November (15°C), respectively. At this time, food resources for adults, such as the flowers of *Hedera helix* L., *Raphanus* spp., and *Solidago* spec., are still present, though getting scarcer. The laboratory data were not supported completely by the data from the semi-field experiment, as egg-laying stopped in the semi-field experiment on 26 September, approximately 2 weeks before the expected date that the first adults would enter diapause.

The date towards the end of March that was calculated for the termination of diapause is only an approximation, as the females of *E. balteatus* in the current study were put into at long-day regimes after only 1 month of diapause. Hence, we do not know if females subjected to diapause conditions for a longer period, would gradually lose their responsiveness to short day-lengths. This happens with many of the insect species of temperate zones that enter diapause. In such species, diapause is often terminated early in the spring when conditions are still cold. Once this has occurred, the insects often enter a quiescent phase, which is regulated mainly by temperature. This ensures that synchronization of insect activity with the weather conditions that occur in early spring is improved even further (Tauber et al., 1986). However, we consider that our results indicate that by March, *E. balteatus* is capable of oviposition and that photoperiods serve the function of controlling the termination of diapause. A further synchronisation by temperature seems likely, as the dates reported in the literature for finding the first eggs of *E. balteatus* have, for various years and locations, varied from mid-March to mid-April (Groeger, 1993; Salveter, 1996) and from mid-April to the beginning of May (Krause & Poehling, 1996). Tolsgaard & Bygebjerg (1991) collected two females in mid-March and could induce egg laying after some days at 20°C and after feeding. This example indicates that diapause was indeed terminated in March as calculated. By that time of year, flowering trees of *Alnus* spp., *Corylus avellana* L., and *Salix* spp. are producing large quantities of pollen, the protein source that hoverflies feed on to develop their eggs (Schneider, 1969).

In Central Europe, populations of *E. balteatus* reach peak numbers during a short period sometime between mid-July and the end of August. The numbers of this hoverfly then decline drastically, which leads to only low numbers being present in mid-September (e.g., Verlinden & Decleer, 1987; Salveter, 1996; Krause, 1997; Hondelmann, 1998). If

this rapid decline can be considered to mark the departure of the hoverflies that migrate to the Mediterranean region (see Aubert et al., 1976; Gatter & Schmid, 1990; Krause, 1997), only the low numbers of hoverflies that remain are subjected to conditions that induce diapause. This would imply that there is a temporal separation between 'migrants' and 'residents'. However, we still do not know what triggers migration, what proportion of the population is involved, and whether migration occurs every year. Dingle (1996) called this type of intra-population variation, which has been reported for several species, "partial migration". For example, in the dragonfly *Anax junius* (Drury) (Odonata: Aeshnidae) a portion of the population is non-migratory and overwinters locally as larvae, whereas the main part of the population migrates to hibernation areas. These so-called migratory and non-migratory cohorts are sympatric, show only slight morphological and physiological differences, and cannot be distinguished by genetic markers. Therefore, it is not clear if this phenomenon is based on genetically determined pathways of development, or simply an expression of plasticity (Freeland et al., 2003). In migratory birds, breeding experiments showed there was a strong genetic component, although influences of the environment were also detected (Dingle, 1996).

In late summer, almost the whole population of *E. balteatus* appears to migrate to the Mediterranean region. These southbound migrations have been observed frequently (see Gatter & Schmid, 1990). In contrast, 'resident' individuals of *E. balteatus* are difficult to find in autumn and we know of only four papers that describe overwintering sites of this hoverfly (Kula, 1982; Tolsgaard & Bygebjerg, 1991; Wolff, 1996; van Grunsven, 1999). Sarthou et al. (2005) reported about catching overwintering stages (active adults) more frequently in south-orientated forest edges with rich flower stands. Although we searched carefully in the types of site in which this hoverfly is supposed to overwinter, such as in crevices in bark and walls including ones covered with ivy, and open sheds (see chapter 3.5.1.2), we failed to find any adults during two winters. Given that successful

overwintering is possible, we surmise that other reasons must account for our lack of success. The first is that only low numbers of *E. balteatus* residents overwinter locally and so they are indeed hard to find. Actually, it is possible that *E. balteatus* even overwinters in aggregations as reported from other insect species (e.g., in Coccinellidae, Chrysomelidae (Coleoptera), and Apidae (Hymenoptera); Danks, 1991; Davenport 1992; Leather et al., 1993). This behaviour is called "shelter aggregation" and possibly attributed to relatively low number of suitable overwintering sites (Danks, 1978, 1991). It is especially useful for animals with aposematic colours such as Coccinellidae since aggregation increases the effect of this colouration and therefore reduces the risk of predation (Klausnitzer & Klausnitzer, 1997). Therefore, it may be also useful for Syrphidae since in this family many species (*E. balteatus* included) are believed to be mimics of Hymenoptera (e.g., Howarth & Edmunds, 2000). In adult-overwintering Syrphidae, smaller aggregations are reported from crevices in walls or caves for Eristalinae (e.g., Feldmann & Rehage, 1966; Brugge, 1980). For *E. balteatus* one report from the Netherlands was published (van Grunsven, 1999) about an overwintering aggregation of females in a hollow of a decaying oak log (described as hibernation site for *Carabus* beetles). Shelter aggregation is not reported in hoverflies overwintering as larvae or pupae (Röder, 1990; Barkemeyer, 1994; Hart & Bale, 1997a).

The second reason for our lack of success is that the hiding places of *E. balteatus* are still unknown. The third reason is that the adults may only use 'hiding places' when conditions become harsh. The first possibility might also be explained by insects that develop too late to migrate southwards and so are 'forced' to overwinter locally, which results in some of them managing to enter diapause. The observed flight activity under mild winter conditions, even as low as 7°C, together with *E. balteatus* adults being caught during the winter months in yellow pan-traps sited in hedgerows and forests (Krause & Poehling, 1996; see chapter 3.5), points to the third possibility that the insects stay active

during the winter. It is striking that in our laboratory and semi-field experiments, *E. balteatus* did not show any negative phototaxis, a behaviour that is typical for animals using shelter in winter (Danks, 1978; Leather et al., 1993).

Compared with another common syrphids, *Eristalis tenax* (L.), which is frequently found overwintering (see Barkemeyer, 1994 for a comprehensive literature list), however, a robust explanation of how *E. balteatus* passes the winter is still required. Particularly, if protected shelters and some thermal buffering are assumed as essential for successful overwintering even for more cold-hardy animals (Danks, 1978; Duman, 2001; Leather et al., 2003; Turnock & Fields, 2005). Moreover, *E. balteatus* being active during the winter months seems surprising, as food is not available and so the risk of energy depletion is high. This is especially attributed to flight activity that is accompanied by very high energy consumption (e.g., Gewecke, 1995). Other insect species that are active during the winter and are in diapause use stored energy and/or do have the possibility to feed (e.g., aphids sucking plant sap; caterpillars feeding on host plants; predatory species (carabids, spiders) still hunting; winter moths of the subfamily Cuculiinae fly during the whole winter and feed on flowers and later on sap from wounds in tree stems (Heinrich, 1987; Block, 1990; Leather et al., 1993). Conversely, *E. balteatus* has no possibility to feed (at least in the northern and central parts of Europe) and we cannot understand the advantage to *E. balteatus* of being active during the winter months. Nevertheless, this behaviour is also observed in other species as for example in *Inachis io* L. and *Aglais urticae* L. (Lepidoptera: Nymphalidae), both adult overwinterers in sheltered sites with considerable cold-hardiness, that are feeding (if the possibility is given) and flying in favourable winter conditions (Pullin & Bale, 1989). The authors attribute this behaviour to a not very deep (intense) diapause, allowing this, in opposite to insects being in deep diapause without any movements. In Diptera, winter activity was reported from *Musca autumnalis* DeGeer (Muscidae), also an adult overwinterer using shelters, but without leaving them during

activity (Burks et al., 1997). For *E. balteatus*, the situation in the south of Europe is different, Sarthou et al. (2005) reported about winter active *E. balteatus* in south France, but feeding is possible in this region as flowering plants in winter are available (F. Arrigon, personal communication). If we compare this with the situation in northern Germany, it may be an indication for missing adaptation of *E. balteatus* to winter conditions in colder climates, and it may be a clue that the latitude of northern Germany is a northern border for local overwintering.

Although in the semi-field experiment some females overwintered successfully, hoverfly mortality was high and could be explained only partially by the factors recorded. As the correlation between mortality and the variables studied was not strong, other factors must have contributed to the overall mortality. It is important to remember that most of the adults we collected may not have been in diapause, as only the offspring they produced were actually exposed to the conditions that induce diapause. Other possibilities are that the adults collected may have been infected with pathogenic microorganisms or that we may not have provided them with suitable media in which to overwinter successfully. In several insect species, infections by entomopathogenic fungi (Madeira, 1998; Thomas & Blanford, 2003) or too high energy consumption (Leather et al. 1993; Irvin & Lee, 2003) during mild winters can result in high mortality during the winter period. The correlation of mortality with humidity and duration of experiment in the data from our field experiment may support such a hypothesis (see also table 12).

In the semi-field experiment of Hart & Bale (1997b), adults of *E. balteatus* failed to overwinter even when conditions were similarly mild. However, as in our results, Hart & Bale (1997b) concluded that the duration of the winter period rather than the actual temperature was the most important mortality factor. The differences between the two sets of results might be explained by the origin of the flies. Hart & Bale (1997b) used laboratory-reared individuals, which are generally more susceptible to adverse conditions

than the wild (feral) flies that we used (e.g., Bartlett, 1985; Nunney, 2002). Some of our flies were in diapause and so were expected to acclimate better to the ambient temperatures found under field conditions. The relation between mortality and duration of the winter in the experiments of Hart & Bale (1997b) may also indicate energy exhaustion, through increased consumption of reserves as a result of not being in diapause. Differences due to strains or races seem unlikely, as no genetic differences were found between the German and UK populations of *E. balteatus* (Hondelmann et al., 2005).

### 3.4.2 Cold-hardiness and Cryo-protectants

Cold-hardiness is the ability of an organism to survive at low temperature (Leather et al., 1993). There are several indices to measure cold-hardiness; the most common ones are the supercooling point (= SCP) and measurements of mortality/survival during long time cold treatments (lethal times, e.g., LT50, LT95) (Zachariassen, 1985; Danks, 2006). For a long time, the supercooling point was used as a standard measure for cold-hardiness and appeared to be a convenient method to compare the cold-hardiness in arthropods (Block, 1991; Bale, 1996). With the discovery that animals can die long before reaching the SCP, i.e., before freezing (Baust & Rojas, 1985; Bale, 1996; Bale, 2002) and additionally due to the high variability of the SCP, depending for example on amount of ice nucleating agents, cryo-protectant content, developmental stage, age, season, and especially cooling/thawing rates in the experiment (e.g., Salt, 1966; Baust & Rojas, 1985; Knight et al., 1986; Leather et al., 1993), its usefulness and especially its ecological importance is now questioned strongly and other measures as for example long-term survival in cold treatments are assumed as superior methods (e.g., Baust & Rojas, 1985; Block, 1991). Therefore, as an exclusive measure, it is not sufficient for the estimation of cold-hardiness and today it is often only used for the determination if an animal is freeze tolerant or freeze intolerant (=

freeze susceptible or avoiding; i.e., the ability of an organism to tolerate the formation of ice in the body or not). However, the interpretation still has to be careful.

In *E. balteatus*, it is already known that it is a freeze intolerant species (Hart & Bale, 1997b) with relatively low supercooling points (in the range of -13°C for not acclimated males and –8°C for acclimated females), indicating this. In freeze intolerant species, the freezing of body fluids is generally lethal (which is proven for *E. balteatus*), therefore in freeze susceptible species, several strategies evolved to achieve supercooling (i.e., the ability to keep body fluids in a liquid state during subzero-temperatures) and to avoid freezing.

Freeze tolerant species on the other hand can cope with some freezing in extracellular compartments (and also developed various appropriate adaptations that are not discussed here in detail). These species generally have higher SCPs, in order to induce the formation of extracellular ice very early and in this way to have sufficient time to make necessary metabolic adjustments and to minimize osmotic shock (Storey & Storey, 1996; Turnock & Fields, 2005). Ice formation in freeze tolerant species is promoted both by internal and external ice nucleating agents such as gut content, bacteria, proteins, and crystalloid inorganic compounds (Storey & Storey, 1996; Lee & Costanzo, 1998). In opposite to this, many freeze intolerant species can remove or mask ice-nucleating particles and lose or at least depress ice-nucleating activity, so that additional supercooling capacity is achieved. The gut is considered as the most important location containing external ice nucleating substances. Consequently, the “clearing of the gut” is considered as one major action in the cold hardening process, in order to remove remains of food, faeces, and bacteria that contain or act as ice nucleators; conversely internal ice nucleators in the haemolymph can be masked by thermal hysteresis proteins (= antifreeze proteins) (Block, 1991; Danks, 2006). Although the significance of the view that all freeze intolerant species have to empty their gut is in question, as several contradictory

observations are reported (Baust & Rojas, 1985), it is remarkable that in all experiments *E. balteatus* adults (males and females) did not empty their guts, but fed on pollen and sugar up to temperatures of 0°C. However, we do not know if this behaviour is attributed to a missing adaptation to low temperatures (i.e., a reduced supercooling ability) or to other reasons (see page 105).

A further important starting point of freezing is external moisture (as droplets on the integument) that can induce ice nucleation in the haemolymph when freezing ("inoculative freezing"). Freeze intolerant species avoid this by using sufficient dry hiding places or by producing own shelter (e.g., tight cocoons). For example in the butterflies *I. io* and *A. urticae* (Lepidoptera: Nymphalidae), the hazard of inoculative freezing is assumed as the main reason to use dry shelters (e.g., sheds), as these species generally can supercool sufficiently, but the supercooling ability can be reduced strongly by inoculative freezing (Pullin & Bale, 1989). This emphasizes again the importance of shelter in such animals. With reference to *E. balteatus*, both Kula (1982) and van Grunsven (1999) (see above) reported apparently about unlikely overwintering sites, as overwintering in leaf litter and open hollows implies unprotected overwintering and the danger of inoculative freezing (actually, van Grunsven thought that the found specimen were drowned). Additionally, in the temperate climate of northern Germany with rare snow cover (a very effective thermal buffer (Bale, 1991)), the temperature on the soil surface is lower than the air above, due to nocturnal radiant cooling and cold air drainage (Bale, 1991), making Kulas' observation questionable.

Another trait to increase supercooling is the production of cryo-protectants working as antifreeze substances. The main mechanism of action is the reduction of the melting point of water, and hence the freezing point by osmotic effects and by the colligatively function of the cryo-protectants for which high numbers of solute molecules

are needed. Therefore, low molecular weight compounds such as polyhydroxy alcohols (= polyols) or sugars are synthesized and then accumulated. Besides, several other functions of cryo-protectants such as membrane and protein stabilization are reported (Block, 1990; Danks, 2006). These metabolites are detectable in many but not all species that can supercool. The most common cryo-protectants are low molecular weight polyols such as glycerol, sorbitol, several sugars (e.g., glucose, trehalose), and metabolites with higher molecular weight such as proteins and glyco-proteins (Duman, 1982; Sømme, 1982; Block, 1990; Bale, 2002). Insects often use more than one of these substances in their supercooling strategy in order to avoid toxic effects of high concentrations of one compound and accumulate them after induction of the cold hardening process. Triggers for the production of cryo-protectants are temperature or photoperiod or a combination of both, respectively (Storey, 1984; Bale, 2002); often an exposition of the insects for several days at low temperatures (5°C and lower) is sufficient (Storey & Storey, 1990; Leather et al., 1993), indicating that our experimental design was adequate to induce a possible cryo-protectant production in *E. balteatus*.

The cryo-protectant concentrations can vary largely between species and can reach up to 5 mol/l (Baust & Rojas, 1985; Block, 1990), although Block (1990) reports 0.4 - 0.6 mol/l as usual concentrations in overwintering, supercooling insects (equivalent to 3-6% of body fresh weight, but exceptionally more than 25% (Asahina, 1966)). Body fluids in the latter insects can supercool up to –50°C as a lower limit in highly cold-hardy insects (e.g., Turnock & Fields, 2005).

The results of the experiments to determine the sugar and polyol content of *E. balteatus* females show that only approximately 1% of the above mentioned usual cryo-protectant amounts were found in the highest concentrated substances (i.e., fructose and glucose). Only if the content of all detected metabolites is summed up (which is from a biological point of view surely wrong), the substances amount to approximately 0.3 - 0.4%

of body fresh weight in the cold treatments. This is still less than in the control treatment with ca. 0.6% polyol and sugar content in the body fresh weight of *E. balteatus*. Accordingly, there were no significant accumulation of sugars or polyols detectable induced by the various treatments (i.e., constant and/or variable cold temperatures in combination with/without diapause and/or two acclimatisation regimes), but decreases. Therefore, the results suggest that *E. balteatus* females do not synthesize any cryo-protectants, as there was no significant increase in any of the examined candidate compounds. However, it is surprising that content of glucose and fructose is relatively high; this was likely caused by a contamination of the samples with gut content (individuals were feed with sucrose), which was not completely removed by the cleaning step in the experimental protocol. The significant decreases in some of the substances (e.g., fructose, glucose) in the cold treatments compared to the control, can be attributed to metabolic reasons, as the experimental flies were under the threshold temperature for metabolic activity (Hart et al., 1997) and therefore consumed their fast-energy reserves, unveiling as decreasing concentrations of sugars and polyols.

Irrespective of the above aspects, *E. balteatus* is supercooling to some degree as our results and the results of Hart & Bale (1997b) suggest. From our results in the lethal time experiments, supercooling capacity can be deduced from the survival at subzero temperatures, because no freezing occurred in the surviving animals. For example 50% survival was observed in diapausing and acclimated females after ca. 100 h at –5°C and ca. 60 h at –10°C (the interpretation of the latter has to be careful as finally none of the flies from the –10°C treatment laid eggs; see chapter 3.3.2). Hart & Bale (1997b) measured supercooling points in acclimated and non-acclimated males and females and found supercooling capacity to some extent (see page 97). The detected supercooling capacity can be attributed to various reasons: First of all, all body fluids contain several

dissolved molecules such as salts, soluble proteins, and sugars, that increase the supercooling capacity to some degree compared with pure water. Storey & Storey (1983) reported that a 0.9% sodium chloride solution has *per se* a supercooling point of approximately –18°C. Additionally, there is a significant relationship between the volume of a fluid and the nucleation temperature: the smaller the volume of fluid is, the higher is the supercooling capacity (e.g., 1 µl of water has a freezing temperature between –16° and –30°C, that increases with increasing volume) (Angell, 1982, in Baust & Rojas, 1985; Lee & Costanzo, 1998). In insects, fluid volumes in the range of several microlitres are typical amounts in their body compartments, implying substantial supercooling ability of tissues and compartments. Both factors can explain the observed supercooling capacity of *E. balteatus*. Additionally, some authors assume that species with some supercooling capacity but with low or missing cryo-protectant production are able to produce thermal hysteresis proteins (e.g., Block, 1982). However, until now it is not known if *E. balteatus* is producing antifreeze proteins, but is seems very unlikely as in the order Diptera until now, no species was found using this type of anti-freeze protection, although Diptera are the predominant insect group in the arctic and a considerable number of species were tested (Duman, 2001).

To summarize, the supercooling capacity of *E. balteatus* can be explained by other causes than anti-freeze substances (as the fluid volume - nucleation temperature relationship and normally occurring soluble substances) and it is unlikely that other not recorded cryo-protectants or thermal hysteresis proteins are produced.

With regard to the above conclusions it is surprising that in the lethal time experiments both acclimatisation and diapause did increase the survival of adult *E. balteatus* compared with non-diapausing and non-acclimated flies. But this is only statistically significant up to 0°C. At –5°C, there is only a tendency that acclimated and

diapausing/acclimated females can survive for a longer time. In the 5°C experiments, non-diapausing and acclimated flies had the shortest survival times (actually even shorter than in the control treatment at 21°C), although this temperature was in the range of non-lethal temperatures. This result was likely attributed to energy exhaustion, as at this temperature *E. balteatus* is at its threshold of metabolic activity (Hart et al., 1997), that is, the flies consumed energy (e.g., animals were active), but they lacked the supply of energy from their reserves. This exhaustion risk was also reported from Hart & Bale (1997b) in their experiments with acclimated but non-diapausing *E. balteatus* in the lethal time experiments as most likely reason for the detected mortality. On the other side, in the experiments of the current study, diapausing individuals at 5°C had no increased or only slightly increased mortality compared to the controls, leading to the conclusion that with diapause adaptations, energy reserves are accumulated, that the flies can use during colder periods or by depressing overall metabolic rate to save energy, respectively. The latter point is problematic, as in this type of diapause the diapause intensity is assumed as not very strong (Pullin & Bale, 1989) as diapausing individuals for example stay active and can feed. Although in normal diapause the metabolic rate is usually depressed (e.g., Storey & Storey, 1990), for reproductive diapause, no published data are available. The slightly increased mortality of acclimated and diapausing *E. balteatus* indicates that the acclimatisation process costs, ergo that there are indeed metabolic changes that need energy. The same pattern was found by Hart & Bale (1997b) where at 5°C acclimated females had a slightly reduced lethal time compared with non-acclimated ones.

In the –10°C experiments, lethal times were again lower and no significant effects of diapause and acclimatisation, respectively, were found, but a tendency that both treatments increased lethal time. Although we do not know the supercooling points of our test insects - they may differ considerable from the data from Hart & Bale (1997b) - the high mortality and the very low lethal times are remarkable, as the temperature was in the

range of the supercooling temperature of *E. balteatus* as determined by Hart & Bale. Therefore, the mortality can be partly a result of freezing, but also other factors may be involved that are discussed later (see page 104). It is interesting that all surviving females out of the –10°C treatments did not lay any eggs, in opposite to the other temperature treatments (only acclimated and non-acclimated females were tested). This indicates that low temperatures can cause "chill injuries" at a sublethal level. This phenomenon is also reported in other insects, as "sublethal cold stress" influencing development, fecundity, and longevity (e.g., Hutchinson & Bale, 1994). This makes the survival estimation in the –10°C experiments somewhat questionable, as surviving without reproduction afterwards is ecologically (and evolutionary) useless. However, it is also doubtful how far the results of laboratory experiments can be extrapolated to field conditions (see page 108). Baust & Rojas (1985) suggested that for such experiments it is generally a necessity to test the reproductive capability as the only accurate test of survival.

Altogether, it is difficult to compare the results of the lethal time experiments with the results of Hart & Bale (1997b), as the lethal times in most of my experiments were evidently higher, sometimes more than two times. Only in a few treatments, (e.g., at 0°C, LT50 of non-acclimated females) there were similar results. This may be attributed to a completely different experimental design, and different origin and quality of the test animals. At least it is notable that in the diapause treatments, lethal times were even higher (Hart & Bale did not have these treatments), indicating again the necessity of diapause adaptations for successful overwintering for *E. balteatus*. If and how far diapause is related with cold-hardiness is still in discussion as there is conflicting evidence. At least in some species diapause seems to be an integral element of cold-hardiness (e.g., for the production of cryo-protectants, behavioural changes, various other metabolic changes), utilizing also the same token stimuli (i.e., photoperiod); in other species, no relation was found (Tauber & Tauber, 1986; Bale, 2002; Danks, 2006). The results for *E. balteatus*

suggest that diapausing individuals are slightly more cold-hardy than non-diapausing ones, although the mechanisms are not known (the conversion of energy reserves (glycogen or fat) to cryo-protective carbohydrates (e.g., polyols) that are then accumulated in the course of the cold hardening process is considered as one major link between diapause and cold-hardiness (Pullin, 1996; Joanisse & Storey, 1996), but cryo-protectants are not accumulated in *E. balteatus*), except for above-zero temperatures, where exhaustion is prevented. Finally, *E. balteatus* shows in all experiments acclimatisation responses that to some degree increase its chance to survive cold periods, but also cost. However, we still do not know anything about the mechanisms of acclimatisation in this species.

The results of the lethal time experiments suggest that males and females can survive at low temperatures for some time. At all temperatures and in all treatments, females had higher lethal times than males (females died later). This may also be owed to the lack of a diapause stage in males as described before. Anyhow, this confirms the results from the semi-field experiment and the results of other studies (e.g., Dušek, & Láska, 1974; Hart & Bale, 1997b) that males of *E. balteatus* are less cold-hardy and are hardly able to overwinter successful.

The foregoing discussion demonstrated that at low temperatures above 0°C, *E. balteatus* mortality is most probably affected by exhaustion. But what about the relatively high mortality between subzero-temperatures and the supercooling point (when freezing occurs that is always lethal in this species) that increases significantly with decreasing temperature? In these long-term experiments, males and females of *E. balteatus* died before reaching the SCP, indicating that not only ice formation is lethal, but that also other factors did contribute to the overall mortality. These factors are addressed in the literature as ‘chill/cold injuries’ or ‘metabolic injuries’ occurring not necessarily at sub-zero temperatures. Long time it was assumed that the mortality above

the SCP was only attributed to spontaneous freezing (since freezing probability increases with time in supercooled liquids), but today other reasons are assumed, too. The mechanisms of prefreeze mortality still are not well understood (Turnock & Fields, 2005), but metabolic disorders are most often considered as primary reason, because these injuries are occurring at temperatures above the insect's freezing point, but below the threshold for normal metabolism and development. The disorders are described as 'disruption of cell metabolism' or 'decoupling normal metabolic processes', including disruption of weak biochemical bonds, "frozen" cell membranes, 'thermotrophic membrane phase transitions', or cold inactivation of proteins (Danks, 1978; Knight et al., 1986; Nedved et al., 1998). Additionally, in prefreeze mortality, the lethal factors are effective during long-term chilling (experiments for determining the SCP are only lasting several of minutes and cannot record them), they are cumulative, but a repair during warmer periods is possible before extensive damage has occurred.

A further cause for mortality during chilling are desiccation injuries. Cold temperatures especially at sub-zero level are accompanied with dry air that can result in desiccation processes (Ring & Danks, 1994; Danks, 2006). The cold periods can last for a long time and overwintering insects cannot avoid this (except through migration before the adverse conditions appear or by selecting more humid, protected shelters) as feeding and drinking is not or only very limited possible (water is frozen, individuals cannot move, or are inactive). During this time, the insect body can lose water through cuticular transpiration, respiration, faeces, and secretion. This water loss must be substituted or minimized by adaptations of physiological processes and/or physical barriers. Many cold-hardy insect species show these adaptations: physical as for example increasing the impermeability of the integument (e.g., by a thicker, waxy cuticle); very important are physiological adaptations such as water reserves (for example metabolic water that is released when energy reserves are used), and the same adaptations that are used for cold-

hardiness, namely accumulation of the same type of metabolites that are used as cryo-protectants (Block, 1996). These compounds work by depressing the vapour pressure of the solution and in this way reduce very effective the water loss (Ring & Danks, 1994). Finally, several insect species can reduce their water content dramatically or withstand water loss, respectively, without problems (desiccation tolerance). This increases on the one hand the supercooling ability (the concentration of the metabolites is higher) and on the other hand, the remaining water cannot be lost or freeze anymore (so–called "unfreezable" or "bound water") (Ring & Danks, 1994). With reference to *E. balteatus,* these adaptations are likely not used, as cryo-protectants are not synthesized and this species is assumed as susceptible to water loss (Röder, 1990; own observations). This is attributed to a relatively thin and weakly sclerotised cuticle (Röder, 1990). Therefore, loss of water may contribute considerable to the mortality of *E. balteatus* during long-term sub-zero temperatures above the supercooling point.

Salt (1961) defined two classifications of cold-hardiness in insects, 'freeze intolerant' (= freezing susceptible or avoiding) and 'freeze tolerant' species. This classification was developed especially for cold climates (i.e., Polar Regions), and is therefore not representing the complete reality in temperate climates, but is somewhat artificial (Bale, 1987). For example some species die above 0°C, others are killed before reaching the supercooling point, and there is much intra-population variability in mortality and supercooling points (Bale, 1996; Danks, 2006). Therefore, Bale (1993, 1996) defined several new classes of cold-hardiness, for a more precise division of the various states. Besides 'freeze tolerance' (now defined as state in which freezing occurs early at ca. –5°C, with only partial freezing of extracellular fluids and high survival from –40° up to –80°C (i.e., lethal temperature much lower than SCP)) and 'freeze intolerance' (now defined as state with extensive supercooling ability and long-term survival with very low or without

mortality in the supercooled state, the SCP is equal to the lower lethal temperature), 'chill tolerance', 'chill susceptibility', and 'opportunistic survival' are defined. 'Chill tolerance' is a characteristic of high to moderately cold tolerant species that supercool and avoid freezing, can survive in this state for relatively long times (several months) but mortality increases with decreasing temperature and increasing time of chilling. 'Chill susceptible' insects have considerable to high supercooling ability, but mortality at sub-zero temperatures above SCP is relatively high, depending on the temperature (between 0° to –5°C high survival rates, but beginning with –5°C, death after short exposures occurs). Finally, 'opportunistic survivors' are insects that can have also extensive supercooling ability, but cannot survive temperatures below their threshold for normal metabolism (generally above-zero temperatures). These insects only survive in good protected sites that provide favourable conditions for the complete period with harsh conditions. In all these new defined classes, the supercooling point is an unreliable indicator of cold hardiness (Bale, 1996).

Hart & Bale (1997b) categorized *E. balteatus* as 'freeze intolerant' species according to the old twofold classification developed by Salt (1961). With this new classification, *E. balteatus* belongs rather to the 'chill susceptible' than to the 'chill tolerant' species, because it has a relatively low SCP (ca. –8° to –12°C), is killed below the SCP (i.e., freezing), can survive considerable time between 0° and –5°C, but has a relatively low cold-hardiness between –5° and –10°C with considerable increasing mortality. Nevertheless, *E. balteatus* is not killed after "brief exposures" to this temperature range, as demanded by the definition (e.g., the LT50 at –5°C is about 100h, at –10°C still ca. 60h). Therefore, the cold-hardiness of *E. balteatus* does not fit exactly in the 'chill susceptible' definition, but seems somewhere between 'chill susceptible' and 'chill tolerant'.

Nevertheless, since most of the results were obtained in the laboratory, it is difficult to conclude from these results to the very complex field situation. There are various reasons for this: The laboratory experiments on lethal times are to some degree "worst case scenarios", as in nature never constant low temperatures are occurring for longer times, but there are continuous changes some in a regular interval (day-night) and others are unpredictable depending for example on the weather. Although the positive effect of fluctuating temperature regimes is somewhat counter-intuitive (see Krause, 1997), in several studies the positive effect of fluctuating temperatures were proofed (e.g., Coulson & Bale, 1996; Nedved et al., 1998; Hanc & Nedved, 1999). Pullin & Bale (1989) also showed that the positive effect depends on the tested species and temperature levels. The results of Kelleher et al. (1987) suggested negative effects of warming (i.e., a reduced cold hardiness) only in non-diapausing specimen of the goldenrod gall fly *Eurosta solidaginis* (Fitch) (Diptera, Tephritidae). One possible reason for the positive effect of fluctuating temperature regimes is that in the warmer phases, the repair mechanisms for chill injuries can start working, whereas at constant low temperatures chill injuries are accumulating and in the end kill the insect (Turnock & Fields, 2005). As additional drawback of laboratory experiments, the cooling and warming rates in these experiments were too high, affecting both cold-hardiness and mortality negatively. This is a general problem of this type of experiments (Baust & Rojas, 1985). In the field, rates of cooling and warming are much slower (e.g., Bale, 2002; Sinclair et al., 2003) and can be buffered further in adequate hibernaculas (see above discussion). In laboratory experiments, rates of 1°C $min^{-1}$ are generally used (Salt, 1961; Baust & Rojas, 1985), but in the field, rates of 0.05° and 0.1°C $min^{-1}$ are ecologically relevant (Bale, 2002). Sinclair (2001) reported even slower cooling rates during microclimate recordings in New Zealand. Nevertheless, such slow rates are difficult or by no means to realize in laboratory experiments.

Burks et al. (1997) reported that in laboratory reared flies (*M. autumnalis*) the acclimatisation is generally not optimal compared to naturally acclimated individuals, which results in lower cold hardiness. Finally, also unusual issues can lead to divergent results between laboratory and field experiments. For example, Kim & Kim (1997) found increased supercooling ability of laboratory-reared larvae of *Spodoptera exigua* (Lepidoptera: Noctuidae) compared to specimen collected in the field. The authors attributed this to increased bacterial contamination that functioned as additional ice nucleating agents in the feral larvae, which were absent in the laboratory culture.

If we compare mortality of *E. balteatus* in the laboratory experiments (lethal time experiments) with the semi-field experiment, it is clearly visible that in the laboratory the mortality is significantly higher. For example in the lab experiments at 5°C over 10% mortality per week was recorded and after 14 days 30% mortality per week (acclimated and diapausing females), at 0°C already over 20% mortality per week with strong increasing rate during the experimental course was recorded. This trend was increasing with decreasing temperatures. Compared with the semi-field experiment in which between 0% to maximum 7% mortality per week was recorded over the complete time, apparently all the laboratory individuals suffered high mortalities, demonstrating the "worst case scenario" of this type of experiment. In the experiments of Hart & Bale (1997b), a similar pattern between laboratory and field trials was observed. However, "real" field data of microclimate conditions (esp. cooling rates, temperatures, and humidity) and the mortality that natural acclimated feral *E. balteatus* experiences, are still lacking.

As conclusion from the results of this study and the results of Hart & Bale (1997b), *E. balteatus* is able to survive low temperatures up to –5°C, if these are experienced not too long, colder temperatures up to –10°C are only tolerable during relatively short time periods (several hours), below this fairly accurate limit, instantaneous freezing and death

occurs. If this is compared with the climate of Lower Saxony, *E. balteatus* can of course cope with the average monthly temperatures that are all positive (lowest temperature is 0.6°C in January as daily mean), but the situation is different if we look at the "frost days" (days in which the average minimum temperature is below 0°C) that add up to in average over 70 days and about 20 "ice days" (days in which the average maximum temperature is below 0°C). Additionally the lowest temperatures are in the range of –25°C for this region (all climate data are 30-year-means for Hannover-Langenhagen and Hildesheim from Müller-Westermeier, 1996). Then it is obviously that this species can only overwinter in protected shelters with sufficient protection against longer cold periods, short cold snaps and inoculative freezing. These shelters are necessary in particular to reduce freezing mortality, nevertheless considerable mortality by chill injuries can be expected also (though this mortality might be not as high as in the experiments, see discussion above). Additionally, other mortality factors such as predation, diseases, and other abiotic factors that are not considered here, add to the mortality. An example how overwintering may take place in *E. balteatus* is the adult overwinterer *Musca autumnalis* in the US (Kansas). This fly has a similar overwintering strategy and cold hardiness as *E. balteatus* (SCP around –15°C, freeze susceptibility, adult diapause with activity and feeding; mostly females overwinter, high mortality during overwintering, acclimatisation enhances cold hardiness) and long time it was assumed that overwintering in unheated shelters in the colder parts of North America is not possible (Burks et al., 1997). This species overwinters in protected hibernacula such as unheated barns and houses, where it faces average temperatures from 0° to –5°C, but also longer periods of –10°C can occur; minimum temperatures in the shelter rarely reach –25°C. Burks et al. (1997) found considerable microclimate differences at different locations in the shelter, with warmest sites in cracks and crevices in an additionally protected wall at the south side of the building. The field temperatures were always colder than these sites, but warmer than

other parts of the building without radiation heat. The shelter reduced also daily temperature fluctuations greatly compared with the field. The flies used the crevices and crawled deeper into them with decreasing temperatures. Although natural mortality was not recorded, in bioassays they found mortality due to freezing and chilling injuries, the latter with increasing importance during the observation period. These results suggested the necessity of good protected hibernacula for the overwintering success in this species. The biological importance of surface warming through solar radiation, heat storage and the retarded release to the environment is known for along time (e.g., Stoutjesdijk, 1977; Danks, 1978) and, though little is known about overwintering habitats of *E. balteatus,* the results of this study and of Hart & Bale (1997b) make it likely that *E. balteatus* uses similar hibernacula that buffer some or most of the threats of cold temperatures likely with the help of stored radiant heat of the walls, good buffering and protection against cold temperatures and moisture. That *E. balteatus* uses radiant heat to warm up is evident due to its darker body colour that adults have when pupal development takes place during cooler temperatures (Holloway et al., 1997). Additionally, Sarthou et al. (2005) reported that *E. balteatus* in south France prefers during overwintering the south sides of forests edges, which they attributed to increased insolation and temperature at these sites.

For *E. balteatus,* there even might be a trade off between sufficient cold protection by shelters and early heating up in spring, as the best protected shelters heat up most slowly and insects using this type of shelters become active significant later than insects overwintering in exposed sites (Leather et al., 1993; Danks, 2006). This pattern can be of importance for *E. balteatus* in terms of resource partitioning between different aphidophagous predators that Dixon et al. (2005) and Dušek, & Láska (1974) interpreted as relatively fixed “temporal attack sequences”, i.e., every species has its temporal niche in spring in which it can exploit aphid colonies with reduced competition within the aphidophagous guild and in which syrphids are the first that attack (actually Dixon et al.

(2005) assumed a phylogenetic constraint and not a response to competition and/or intraguild predation that induced this phenomenon).

Finally, in insects using good insolating hibernacula, an increased resistance against chill injuries would be more important than an increased supercooling capacity. The mechanisms to avoid non-freezing cold injuries have received only little attention compared to freeze injuries (Turnock & Fields, 2005). The importance of suitable shelters for natural enemies and by what means it is possible to promote them was examined in several studies (e.g., Sengonca & Henze, 1992; Thomas et al., 1992; Thierry et al., 2002). The results of these studies suggested that natural enemies (in the studies Coleoptera and Neuroptera were used) have well defined overwintering requirements and that these can be exploited for example in the management of field boundaries (e.g., by offering suitable shelters).

# 4. Final Discussion

In *E. balteatus*, the probability for successful overwintering in central and northern Europe was considered generally as not very high or only possible in mild winters and its importance for population development with high numbers in summer low, respectively (Stubbs & Falk, 1983; Verlinden & Decleer, 1987; Röder, 1990; Torp, 1994). This point of view was supported by the results of Hart & Bale (1997b) who found only low cold hardiness in UK populations of *E. balteatus*. Conversely, the high mobility and the numerous reports about its migrations (see introduction), led to the opinion that at least in Europe all (sub-) populations are connected by migration and dispersal events, respectively. Long-distance migration, which is postulated to be seasonal, i.e. regular occurring between southern (Mediterranean) and central/northern European regions, should start in late summer to Mediterranean Europe for overwintering and from April to June northbound to regions with colder climates (Aubert et al., 1976; Pollard, 1979; Gatter & Schmid, 1990). Consequently, low cold hardiness was considered as one of the most important reasons for seasonal migration between southern and northern parts of Europe. Only some authors questioned this view and preferred a local overwintering, by autumn fertilized and cold adapted female adults (e.g., Salveter, 1996). The only agreement in all studies is, that males are relatively short-lived and neither migrate nor overwinter successfully as residents.

In summary there is now a huge body of records, observations and papers giving arguments against and for migration and local overwintering, respectively, therefore the following paragraphs should compile and consider the pros and cons of both strategies as well as some neglected topics with regard to both strategies.

**Migration**

Two long-term studies and many occasional observations support the view of southbound migration (Aubert et al., 1976, Gatter & Schmid, 1990, and references therein), and indications for migration over sea/offshore exist (e.g., Lempke, 1962; Heydemann, 1967; Svensson & Janzon, 1984; Schmid, 1999; Kehlmaier, 2002). The study of Hondelmann et al. (2005; see chapter 2) revealed by means of genetic markers that European populations of *E. balteatus* are genetically very similar, indicating extensive gene flow, and no isolation of subpopulations across Europe. Nevertheless, until know, there is no definite proof for a regular, seasonal, and bi-directional migration. Indirect evidences however are observations of the regular disappearance of huge numbers of *E. balteatus* within a short time period in late summer in central Europe (e.g., Krause, 1997; Hondelmann, 1998) and the following arrival of migrants on typical routes used by birds to cross specific passes at the Alps, Schwäbische Alp, and the Pyrenees (Aubert et al., 1976, Gatter & Schmid, 1990). On the other hand, until now, conspicuous northbound migration was never documented and again only some observations support such a directed seasonal movement: Kehlmaier & Martínez de Murguía (2004) reported about a strong increase in numbers in May/June in their Malaise trap catches in northern Spain (Navarre) that they attributed to presumably northward travelling *E. balteatus.* In Scandinavia, *E. balteatus* is occurring not before June or even later, indicating a relatively late arrival of immigrating flies from southern areas (Gatter & Schmid, 1990; Torp, 1994). Krause (1997) suggest various possible explanations for the lack of perceptions of northbound migrations (e.g., low and therefore difficult to observe numbers of migrants; other, unknown migration routes; a geographically more spreaded migration without conspicuous peaks), which are all still speculative.

However, it is questionable whether aggregations of high numbers of dispersing insects only result from long distance migration events. Nielsen (1964) stated that

temporary mass aggregations can result from local synchronized emergences and be misinterpreted as migrations. Short-term aggregations and synchronized movements can occur for example when high numbers of offspring develop to adults in then food-depleted habitats. This type of migration then simply aims in seeking for new habitats, e.g., for founding a new generation. Krause (1997) was the first who related the observations of Nielsen (1964), to supposed migrations of *E. balteatus* populations. This species can develop in very large numbers for example in cereal aphid colonies and in particular in wheat during mid-summer (June) in central Europe. Salveter (1996) calculated $10^5$ *E. balteatus* pupae per hectare in heavily infested winter wheat stands, which develop relatively synchronized. After hatching, adults have to seek for pollen and nectar sources and later for new aphid colonies and disperse therefore in surrounding areas. This phenomenon is also the basis of so-called spillover effects in agro-ecosystems, the movement across agricultural-to-natural habitat edges by subsidized natural enemies developing within fields. Immigrants then can affect the fauna of natural habitats (e.g., Rand et al., 2006). It is unclear which or how many of the migration reports of hoverflies can be attributed to this phenomenon.

An additional weakness to support the migration theory is that nearly nothing is known or researched about specific adaptations and behavioural changes necessary for successful long-term flights of syrphids. It is for example astounding that the diapause induction, which is generally also a prerequisite for migration (in terms of energy storage and interrupting reproduction), is induced in northern Germany after the assumed migrants have left that region. However, most possible adaptations and almost all physiological aspects (e.g., flight muscle build up, sensitivity for migration triggering signals, mechanisms of central nervous and/or hormonal control of the migration behaviour, possible genetic determination of migration behaviour) are still not investigated. Especially, further knowledge about factors which determine the timing of migration are

important, as - similar to diapause for hibernation - migration has to be synchronized with the season. In the semi-field and laboratory experiments in this study, indications of directed flight behaviour (e.g., flies accumulating in cage edges) or even 'migratory restlessness' ("Flugunruhe"), a typical behaviour of birds and insects (e.g., Dingle, 1996; Rodríguez-Clark, 2004), could not be observed.

**Cold hardiness**

Experiments to study the cold hardiness and survival of *E. balteatus* revealed only low cold hardiness of acclimated and both acclimated and diapausing females (Hart & Bale, 1997b; this study). First semi-field experiments to confirm winter survival of *E. balteatus* in northern Europe by Krause (1997) and Hart & Bale (1997b) were not successful. As a consequence of their results, Hart & Bale (1997b) considered local overwintering in the UK as impossible and explained winter findings of active adults by the use of artificial overwintering sites such as greenhouses. Even diapause induced individuals suffered from high mortality in our laboratory and semi-field experiments. Except the diapause stage, no further adaptations for cold hardening and overwintering could be found. However, occasional observations of active adults in late autumn, winter, and spring are well documented in northern and central Europe (e.g., Goffe, 1934; Schneider, 1948; Veltman, 1977; Toth, 1985; Hastings, 1988; Schier, 1988; Wolff, 1990; Salveter, 1996; Krause & Poehling, 1996; Krause, 1997; own observations) and were the main arguments for local overwintering. Additionally, with trap plants (Krause, 1997) in early spring and yellow pan traps in winter (Krause 1997; this study) early oviposition and active adults, respectively, are detectable. The caught winter active females were all in reproductive diapause.

How significant are the experiments and observations? Generally, all laboratory experiments concerning cold hardiness are 'worst case scenarios' due to continuous low

temperatures, too high experimental cooling/warming rates, and an imperfect (compared with natural conditions) acclimatisation (see page 109). This explains at least partly the high mortality. However, the long survival of *E. balteatus* females in our semi-field overwintering experiment suggests that successful hibernation of adapted females is on principle possible even in the north, if the conditions (diapause induction, appropriate shelters, acclimatisation in autumn, sufficient food supply in late summer, healthy animals) are adequate and temperature flutuations moderate. A major drawback for further evaluations and hibernation experiments is that the natural overwintering sites are still unknown. The rare evidence of such hibernacula with overwintering individuals (Kula, 1982; Wolff, 1990; Tolsgaard & Bygebjerg, 1991; van Grunsven, 1999) was used as counter-ecidence of local overwintering (e.g., Hart & Bale, 1997b), but this phenomenon can also be explained by other reasons (see discussion in chapter 3.4.1). The suggestions of Hart & Bale (1997b) with protected sites such as greenhouses as main hibernation sites are not very convincing, because during winter active residents were often found far away from such places and there is only little give the opportunity for moving such distances at low temperatures. Additionally, in greenhouses the regularly pest control makes survival difficult, as *E. balteatus* has to pass through the complete life cycle during this time and is highly susceptible to pesticides in all stages (e.g., Drescher & Geusen-Pfister, 1991; Colignon et al., 2003).

**Mediterranean region**

In general, arguments for leaving the Mediterranean region during summer focus on the assumption that during this period the Mediterranean basin are generally unfavourable for *E. balteatus* and other syrphids, because adult flies need flower stands with attainable nectar and pollen sources and larvae prey on aphids. Additionally, adults of *E. balteatus* are only weakly sclerotised (Röder, 1990) and prefer relatively humid and shady

conditions. All these resources and conditions become scarce in this period (e.g., Dunn et al., 1977; Gatter & Schmid, 1990) and therefore should force migration to the north. However, Salveter (1996) questioned such simple considerations and the significance of migration and argued that throughout the time of southward migrations of this species, the Mediterranean vegetation should be still dry and aphids not available. On the other hand, when the occurrence of large numbers of *E. balteatus* in summer in Scandinavia is indicating an expansion to the north, the aphid population densities in this region are still low, whereas aphids become at this time abundant in central Europe. Considering these prerequisites, why should migration take place at all?

Both points of view seem too simple in reality, as the Mediterranean region cannot be considered as a homogenous dry area during summertime. Although in summer wide areas of the Mediterranean are unfavourable environments for *E. balteatus* due to the aridity, heat and lack of resources, there is a gradient with unfavourable conditions becoming more extreme towards the south, where summer drought can last for 5–6 months instead of 2 months typically recorded in the north of the Mediterranean region (Blondel & Aronson, 1999). Hence, there are several reports that in the northern, as well as higher and cooler parts of the Mediterranean regions, flowers are available throughout the year, which are visited by syrphids including *E. balteatus* (e.g., Herrera, 1988; Bosch et al., 1997; Kehlmaier & Martínez de Murguía, 2004). For example, plant species such as *Sonchus tenerrimus* L. (Asteraceae), *Andryala integrifolia* L. (Asteraceae), and *Foeniculum vulgare* (Miller) (Apiaceae) are blooming in summer and visited by syrphids (Bosch et al., 1997). Conversely, *Lobularia maritima* (L.) Desv. (Brassicaceae) is in bloom almost the entire year and is visited and pollinated in summer mainly by bees and ants (Hymenoptera), but in autumn and winter by Diptera (including syrphids) (Bosch et al., 1997). Herrera (1988) reports that in a southern Spanish scrub community, *E balteatus* visits mainly *Daphne gnidium* L. (Thymeleaceae), *Smilax aspera* L. (Smilacaceae) and

*Calluna vulgaris* (L.) Hull (Ericaceae) that have their flowering peaks from August to November. These examples show the variety and the differences of habitats in these regions and the heterogeneous responds of insects to this. Finally, Herrera (1988) also reports about a general activity peak consisting of Syrphidae and Calliphoridae in late summer to early autumn. If this can be attributed to immigrating Diptera, then indeed syrphids arrive in Mediterranean regions during their southbound migration (however, in this region scrubs were flowering and syrphids were detected during the whole season). For an extensive analysis, the vast literature about flower phenology and the flower visitor communities of the Mediterranean region needs to be studied exhaustively, although often the insect species are not mentioned in detail.

In the northern parts of the Mediterranean region, also some cold hardiness and supercooling ability is required (e.g., Milonas & Savopoulou-Soultani, 1999), as the winters can be relatively cold with sub-zero temperatures, too. Examples are northern parts of Spain and Italy, more elevated and inland regions with a more continental climate (Rother, 1993). Some supercooling ability to achieve higher cold hardiness in migrant insects is reported (e.g., Larsen & Lee, 1994), if the hazard of cold periods during migration or at the destination exists. In *E. balteatus,* the found supercooling ability and cold hardiness seems to be perfectly fit to the winter conditions of the Mediterranean basin and so this species can overwinter there without any problems. Altogether, these points indicate that similar to northern Europe, parts of the population may not migrate during spring northward but may have the possibility to stay. This entails the question about larvae in this region. It seems relatively clear that *E. balteatus* can complete its development in the Mediterranean region, as in wintertime aphids are available. Although records of larvae in this time are rare but available (S. Rojo, pers. communication), it can also be derived from the genetic similarity of populations from Spain and other European sites and the longevity of this species (as arriving females are to old to fly back, and

therefore the next generation has to do this). However, less is known about a possible summer development of *E. balteatus* and aphid availability during this time. The only report is from S. Rojo (pers. communication), that in northern Spain, larvae of *E. balteatus* are available in July. Generally, it seems that this topic is neglected in this region (S. Rojo; D. Sommaggio, pers. communications).

There are some indications that the genus *Episyrphus* has a more Mediterranean to tropical distribution. In the Indomalayan region (especially India) the genus consists of more than eleven species (Kapoor et al., 1985; Han et al., 1998), in the Australasian-Oceania region approximately seven (Thompson & Vockeroth, 2007), and in the African region (Afrotropic and Paleartic parts of Africa), six *Episyrphus* species were found (Wakkie, 2007). Surprisingly, although it is assumed that *E. balteatus* occurs in Africa with the Ethiopian region as southern border (Dirickx, 1998), in the current checklists (Wakkie, 2007), it is lacking. This may indicate the low abundance of this species in Africa, but the checklists for Africa are certainly not complete. In the Indomalayan region, *E. balteatus* is the most ubiquitous species of this genus and has some economic importance as aphid antagonist (e.g., Hamid, 1983; Kapoor et al., 1985). This may imply that this genus and perhaps *E. balteatus* has its origin in these regions and therefore corresponding climatic demands. *E. balteatus* as most eurytopic species has presumably spread its range most and is still spreading (Kapoor et al., 1985), which is also a support of the migration theory.

As a conclusion, parts of the southern populations of *E. balteatus* certainly can aestivate in warmer climates for example by withdrawing to suitable habitats with continuous food supply and humid microclimates (especially in northern and higher parts of the Mediterranea) and therefore may not contribute to the northbound migration in spring. On the other hand, the found winter adaptations of *E. balteatus* could also be

interpreted as adaptations of a migrating species that has to cope with some cold during migration or at its southern overwintering sites. Therefore, it would be interesting to compare the cold hardiness of overwinterers from the Mediterreanean region with northern overwinterers. According to the available checklists, *E. balteatus* does not overwinter in northern Africa.

**Conclusion**

The results of this study are ambiguous. On the one hand, a relatively low cold hardiness of adult *E. balteatus* was found, although it was higher compared to the results of Hart & Bale (1997b), but the adult diapause stage can allow local winter survival and is necessary for survival at low above-zero temperatures by avoiding energy exhaustion. Nevertheless, sub-zero temperatures above the supercooling point lead to considerable chill injuries that strongly add to mortality with increasing time period and decreasing temperatures. Even if the noticed mortality both in semi-field and laboratory experiments is higher than under realistic conditions (see above), altogether the more severe winter in northern Germany compared to the UK, the missing further metabolic adaptations for overwintering (e.g., cryo-protectants), and the high mortality make it clear that the adaptations of *E. balteatus* to local overwintering in central and northern Europe are poor and local hibernation involves a very high mortality risk. The mortality caused by freeze injuries can certainly be reduced by the use of suitable shelters (which still have to be detected) with sufficient buffering against sub-zero temperatures and too high temperature and humidity fluctuations and by utilising stored radiant heat. However, also chill injuries will contribute to mortality under natural conditions and in shelters. Hence, a successful overwintering of few protected females similar to that found in *M. autumnalis* (see Burks et al., 1997) irrespective of low cold hardiness and high mortality high could be possible (see page 110).

On the other hand, the findings of this study strongly suggest migration of European populations of *E. balteatus*, due to lack of population subdivision, low genetic distances between populations, the very high gene flow rates, and the complete lack of isolation by distance. The high genetic diversity also is a strong argument for northbound migration (seasonal or not), because a population build up in spring only by residents

would result in a bottleneck effect with strongly reduced genetic diversity in central and northern European populations.

Both hibernation strategies entail high risks of perishing. Survival and in consequence, population build-up in the summer likely depends primarily on weather conditions, as the severity of winter mainly for the residents, wind direction and rainfall, or spring drought in the south for migrants. Thus, a double strategy of local overwintering and large-scale migration is likely as it would spread the risks and possibly increases the chances of survival for the population ('bet-hedging'). Hence, in future studies, attempts should be made to quantify the contribution of such migrants and residents to the summer populations of *E. balteatus* from central and northern Europe. An approach to do this could be to develop molecular markers with much more resolution such as microsatellites or sequence analyis (but see Szalanski (1995) and Freeland et al. (2003): both estimate this as difficult or impossible in largely unstructured populations). This method is difficult due to several reasons: migrants and local overwinterers are not distinguishably morphologically, so it is not known what is actually analysed; the numbers of found local overwinterers and presumable of migrants in the Mediterreanean region are too low for satisfactory genetical and statistical analysis. These problems may partly be avoided when collecting directly in the southerly overwintering areas and by collecting flies from interception traps e.g., in the Schwäbische Alp or the Alps. Another approach would be to make use of a tethering technique and a flightmill to compare the flight activity and willingness of the different overwinterers.

**Plant Protection Issues**

Based on our data and those published in the literature, we can surmise the following about the impact of syrphids in regulating cereal aphid numbers in northern Germany. It is usual in this region for cereal crops to become infested with aphids sometime between the

beginning of May and the beginning of June, if as in most years, holocylic development is dominating (Chambers & Adams, 1986; Tenhumberg, 1992). Typical mass development of cereal aphids then can occur between mit of June and early July. *E. balteatus* populations that migrate from the Mediterranean region northbound cannot be expected to arrive before June (van der Goot, 1979) and so would be too late to establish sufficiently high populations at the beginning of aphid gradation in most years. Nevertheless, such syrphids can contribute to the large population of *E. balteatus* that develop later in the season. Local overwintering females on the other hand can lay eggs from March onwards. This would enable their offspring to prey on aphid populations on winter hosts, such as *A. fabae* on *Euonymus europaeus* L. or *Rhopalosiphum padi* (L.) on *Prunus padus* L., and allow a first generation of hoverflies to develop near the cereal fields (Salveter, 1996; Krause, 1997). The next generation could then colonize cereal fields in greater numbers and early enough to reduce cereal aphid numbers. In theory, therefore, there would be a good synchronisation between the development of the aphid and syrphid populations. However, the total numbers of *E. balteatus* that overwinter successfully appears to be very low. Hence, it seems doubtful whether sufficient *E. balteatus* overwinter to make any appreciable impact when the cereal aphid colonies establish in spring. However, the high fecundity of *E. balteatus*, which is about 1000 eggs/female (Ankersmit et al., 1986; Hindayana, 2001), the high prey finding abilites (Bargen et al., 1998) and the exact oviposition even within small aphid colonies reducing the risk of food shortage for the offspring could help to compensate for the low number of surviving females. Furthermore, there is apparently no trade off between fecundity and diapause and cold treatments as our results showed, which is contrary to several other species (Leather et al., 1993), and contrary to the opinion of van der Goot (1979), who assumed that Syrphidae (*E. tenax* and *E. balteatus*) after local overwintering do not reproduce in the spring, but die. The early findings of eggs and larvae on trees and on trap plants (e.g., Krause, 1997) and

evolutionary reasons (how should this behaviour develop and bequeathed to the offspring when local overwinterers become extinct every year?) make this theory very unlikely.

If *E. balteatus* uses both local overwintering and migration to spread the risk of mortality, it could be expected that the relative importance of the two strategies will vary from one year to the next. Both strategies are associated with a high number of risks. Apart from the key factor weather, that influences diapause induction, starting time and success of migration, and winter survival, other determinants (which are also depending partly on variable weather conditions) like natural enemies, fungal infections, or a lack of food during the autumn months are important alike. As a result, the success of migration and local hibernation will differ between years and regions and this will be reflected in the relative composition and by high seasonal and spatial fluctuations of the *E. balteatus* populations. Especially in years when the overwintering population gives rise to high numbers of hoverflies, or when high numbers of migrants arrive early, *E. balteatus* females will be present in sufficient numbers to reduce cereal aphid numbers in northern Germany. When coming back to the initial question how to improve the potential of syrphids to control aphid infestations, it seems quite questionable whether making the landscape adjacent to cereal fields more diverse, will 1) play a key role in improving the synchronisation of *E. balteatus* with the aphid prey of its larvae, or 2) will make the build-up of the spring population of *E. balteatus* more reliable. *Episyrphus balteatus* is a highly mobile species, and so it could overwinter in sites some distance away from cereal fields. However, many of the conclusions about whether beneficial insects moving from one region or locality can impact upon established pest insect populations in another region or locality are contradictory (e.g., Wratten et al., 2003).

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# 6. Annex

### 6.1 A+T-rich region consensus sequence

The consensus sequence was assembeled using Bioedit 7.0.5. The following abbreviations were used: R: G or A; Y; T or C; M: A or C; K: G or T; S: G or C; W: A or T.

TGCTGG CACGAA TTTTGT CAAAAA AGAAAA AACGAA GAAAGG TTGCTT
TTTTTT TCTATT CCATAG CCATCG TCGCGC TGTAGT TCAAAA ATTTTA
TTTAAA CATTTT TTGATA ATTTTT TTCTCT TGATTG TTTTGT CCTTGG
AATTAT TATGGT STTCCT TGTAGA TGTCTC AAAATT AAATAA ATTGTC
ACTCTA TAGTTT YCCTTG CAAAAT CACAAT CGACTG CAATTT TGCTCA
TAGAAA AAAGTG ACGTTA GGTCCA ATTAAT AWTCCC TAAAAT CCCTGT
TTTCTC TTCRAG GATATT TTACCA AAAGGT AAACAA TCTGAT TGGAAT
GTTGAA TGATAA AAAATC TACAAC ATTTTG AGACTC TGAGTC CCGAAA
TTACAA TTTGTT AAGAAA TTTTCA TGAACC CGTGAG CAAAAT TTTATC
GATCGA AGCTGT CATGTG ACTGTC ATCAAA CAGCTR ACTCTG GTTCTA
CTCGTA TTCGTA TTCGTT ACTCGT AATAGT CGACTT GCCTTG CTTTGC
TTTTGG TTTTGG TTTTTG CTTTGC GCGGCG TCTACA AGTCGA CAGAGG
GTGGTT GTTGGG TGAATC TGAATG ATGATG ATGATG ATGTAG GGATTG
TGGACA AAGATA GTTTGA CTATAA TGTCAA AGTTTT GCTGGG GCTCGG
TGTCGA CGAACA CGCATT TTTGTG TGCTCC CCAATT TTCTGG CCARGK
GGKTGG KTTTTT TTWATA ATYCAT TTTTGG CTACTC GCTCGT TTGAAG
AACTTT GTKGTT TTCTGY TCAWAT TTTTGG TTAGAA TACCAA AAAAAA
AGGCAA TTATAA TTGATG TGTAAT ATAATT TAATTC CAAATT ATATAC
GTGAGA TCTGTG GGGCCT CCTCCA CCAACG TTTTTG GTGTTT TTTCTG
TGCGAA ATGTAG ATAGTG CAGTTG ATATTG GACATC ATCGTC AATAGG
ATTGAG RGGTGC TGATGG ATTTAT TGATAT ATTATA ATCATC GACGAC
GACGAC AACGTT TTTGAG CTTCTG TTGAGA AAATAA AAGCGT GTTATC
TCCGTT TTTATT GTACCT ATTTTT GTTGTT GTTTTT GTTTTA TTTATT
TTTTTA TTTCTG ATTACC ATTAAC GGGCGC CGACGA CGACAC AAAGCC
TGGGTA CACAAA AACCTT TTTTCT TTTTTC TTCCGG AGGACG TCATCA
TCAACA TCATTC AAAATG AATATG TTTCCT CATAAA CATGGT AAATAT
GGAAGC ATTTTT TTCAAG AGTACC TATTTA TTTGTT AAGGGA TGAGST
ATTTTT AAATTT TTTTAA ATTTAA TTTTAG AGAATT GGTAAT ATTTGG

ATAGTG TCCAGA TGGTTG ACCCAT TTGAAT GTTTTT TTTTTT GTAATT
TTTATT AATATT ACAGCA AACGTG TAAGGT GTCCTC GTTTTA ACAGTG
TTATTT ATTGAC AAAATT CGTGCC AGCA

### 6.2 Complete list of A+T-rich region haplotypes used for statistical calculations

| Haplotype number | Binary code | Alphanumeric code |
|---|---|---|
| 1 | 01111001101101110101010 01001110 | AAAA |
| 2 | 01111001101101110101000 11101 10 | AAAB |
| 3 | 01111001101101110101011 1001110 | AAAC |
| 4 | 10110100101010111101010 1001110 | BDBD |
| 5 | 01111001101101111101010 1001110 | AAAD |
| 6 | 01111001101101111000010 1001110 | AAAE |
| 7 | 01111001101101110101010 1000111 | AAAF |
| 8 | 01111001101101110100110 1001110 | AAAG |
| 9 | 01111001101101110111000 0001110 | AAAH |
| 10 | 01111001101101110101000 1101110 | AAAI |
| 11 | 01111001110001110101010 1001110 | ABAA |
| 12 | 01111011101101110101010 1001110 | ACAA |

### 6.3 *Zen*-region consensus sequence

The consensus sequence was assembeled using Bioedit 7.0.5. The following abbreviations were used: R: G or A; Y; T or C; M: A or C; K: G or T; S: G or C; W: A or T.

GACAGT CCTCCG ACTATC TTTCTA AGGATA ACAGCT CATTTC ATTTAG
AAAAGA AAACAT TTTACA GTTTCA TATTTT CCAAAC TTATTT TTCAAT
TTTTCG AAAATA CTACAC TATATT ACCATG ATGTCA TTCAAG CAAGAA
TTTTTA AACTAC CCAAGA TCATCA CCACCA CCATAT TCRGCT CATTTA
GAAATG ATGAAT TGCAAT TTYGAA CCAACA ACTGTG ATTGTG AAGAAA
TCCGAG TCACCC TCACCG AATAAG GAAAAG TGCAAG CGAGCA AGAACT
GCATTC AGCAGC AATCAA TTGATT CAGCTC GAGAGG GAATTC CATACC
AACAAG TACCTG TGTCGT CCACGA AGAATC GAGATT TCTCAA CGTCTT
GAACTC TCCGAA AGACAA GTGAAA ATTTGG TTCCAA AATCGT CGCATG
AAGAGC AAGAAA GATGCT GCTCGA GGCATC ACCGAT TACATC AAGTTT
CGACCA AGTTCC GATTCT GGATCA AGTCGT GGAAAT TCAGCA CCAGTT
TCACCA CATCAG AACTTT GGAAGT CCAAAT ATTAAG ATCGAA GCTCAG
AGTGAT GATAAG AGTCAT GATGGA ATTGTA CAAAGA CTCCTG CAGTAC
AGTCCA CAGCGT GAACTT TCAACT CATCAA GTTGTA ACCCCA GTACAA
GTTCAA AACCAG GCACAG CATCAG CAACAG CCCATG CAAACT ACCAAC
TATCAA ACAGTC AACGTA AATGAT AACATG GAAGTT CCACGA TACATT
AATCCT TATCAA ATGCAT AGCAAC TATCCA TCAGCA CCTGTA TCTCAT
CAGCAT CAGACC AGCCAT ATGAAT GCAGGA AATAAT TTTATC TCCCAG
AACTAT ACCACC CCAACC TCTACC GATTTT CCTTCA AGCGAT CGATTG
AACAAT GTCTTC GATCAA TTTATT CCAAAT TTCTTA GACTTT GCAATG
GGCAGT GATTTG ACTTAT GAACCT GTGCAG GCGTAC GATAAC AACAAT
AGCTGC GATCTT TCACCA AATTCA GCAAGT GACGAA AGCTGG GCTTCG
AGTTTT TCCGCA CTACCA TCTGAT ATTGAC TTAACA GCTCAC CGATTG
TTAGAA TTGTAA AACTTT AGTAAA ATATTA TTTTTG TTGATA TTGTAA
ATAAAA TAAGTT TTAAAA

## 6.4 Complete list of *zen*-region haplotypes used for statistical calculations

| Haplotype number | Binary code | Alphanumeric code |
|---|---|---|
| 1 | 1101011011011101011 | AAAA |
| 2 | 1101011011011110011 | AAAB |
| 3 | 1101011011011101111 | AAAC |
| 4 | 1111011011011101011 | BAAA |
| 5 | 1101011011011100111 | AAAD |
| 6 | 1111011011011111011 | BAAE |
| 7 | 1101011011011111011 | AAAE |
| 8 | 1101100011011101011 | ABAA |
| 9 | 1101100011011101111 | ABAC |
| 10 | 1101100011011111011 | ABAB |
| 11 | 1101011011011110111 | AAAF |
| 12 | 1101011011011111111 | AAAG |
| 13 | 1111100011011111011 | BBAE |
| 14 | 1111100011011101011 | BBAA |
| 15 | 1111100011011100111 | BBAD |
| 16 | 1101011100100101111 | AABC |
| 17 | 1011011011011101111 | CAAC |
| 18 | 1101100011011110111 | ABAF |

## 6.5 Publications derived from this study

Hondelmann, P., Borgemeister, C. and Poehling, H.-M. (2005): Restriction fragment length polymorphisms of different DNA regions as genetic markers in the hoverfly *Episyrphus balteatus* (Diptera: Syrphidae). Bulletin of Entomological Research 95: 349–359.

Hondelmann, P. and Poehling, H.-M. (2007): Diapause and overwintering of the hoverfly *Episyrphus balteatus*. Entomologia Experimentalis et Applicata 124: 189–200.

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