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Study of the impacts of increasing temperature on metabolic rates in two macroinvertebrates : *Limnephilus vittatus* and *Aeshna cyanea*

Fuentes Mathilde

Key words : Metabolic rates; Climate change; Temperature; *Limnephilus vittatus*, *Aeshna cyanea;* Macroinvertebrates.

České Budějovice, South Bohemia, Czech Republic.

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Abstract

English version

From this year, the members of the Aquatic Ecology Laboratory in the University of South Bohemia, conduct studies to evaluate the impacts of climate change on freshwater ectotherms. During my internship, I studied specifically the impacts of rising temperature on metabolic rates. We focused our studies on 2 species : *Aeshna cyanea,* a dragonfly larva *and Limnephilus vittatus*, a trichoptera larva.
Metabolic rates are closely linked to respiration rates (main source of energy production). Therefore, experiments realized in laboratory were based on respiration measurements (i.e. oxygen consumed) through different increasing temperatures. Activity rates were also measured in *Limnephilus vittatus* to evaluate if activity was affected by temperature in the same way than metabolic rates.
Those measurements allowed seeing how the two aquatic insects studied coped with changes in temperature by obtaining thermal response curve for both of these insects.
Concerning *Aeshna cyanea*, the results showed an increasing of metabolic rates with temperature.
For *Limnephilus vittatus,* the trials realized allowed discovering an experience effect (or learning effect) : metabolic rates and activity were higher for the first trials (caused by stress) than for following trials (adaptation). This effect masked the investigated impact of increasing temperatures on metabolism and activity.

Key words : Metabolic rates; Climate change; Temperature; *Limnephilus vittatus*, *Aeshna cyanea*; Macroinvertebrates.

French version

Les membres du laboratoire d'écologie aquatique de l'université de Bohème du Sud ont initié en 2017, un projet de recherche permettant d'évaluer les impacts du changement climatique sur des espèces ectothermes aquatiques.
L'objectif de l'étude présentée dans ce rapport a été d'étudier l'impact d'une hausse des températures sur le métabolisme de deux macro-invertébrés aquatiques : des larves d' *Aeshna cyanea*, ou Aeshna Bleue (odonate) et des larves de *Limnephilus vittatus* (trichoptère).
Le métabolisme étant très lié à la respiration (celle-ci étant la principale source de production énergétique), les expériences réalisées en laboratoire se sont basées sur une mesure de la respiration des insectes (i.e. l'oxygène consommé) à travers des températures croissantes. Le taux d'activité a également été mesuré chez les larves de trichoptères afin de déterminer si ce paramètre variait de la même façon que la respiration face aux différentes températures.
Ces expériences ont permis d'observer comment les deux espèces étudiées se comportaient face à un changement précis de température en obtenant des courbes de réponse thermique.
Les résultats concernant *Aeshna cyanea* ont montré une hausse du métabolisme suivant la hausse des températures. Pour *Limnephilus vittatus*, les tests réalisés ont permis de mettre en évidence un effet d'expérience ou d'apprentissage : le métabolisme (ainsi que l'activité) des insectes était plus important lors des premières mesures (dû au stress) que lors des mesures suivantes (adaptation). Cet effet a en partie masqué l'impact recherché des températures sur le métabolisme ou l'activité.

Mots clés : Metabolisme ; Changement climatique ; Température ; *Limnephilus vittatus*, *Aeshna cyanea*; Macro-invertébrés

Preamble : Short report version

Study of the impacts of increasing temperature on metabolic rates in two macroinvertebrates : *Limnephilus vittatus* and *Aeshna cyanea*

Context :

The Aquatic Ecology laboratory at the University of South Bohemia is conducting studies to evaluate the impacts of climate change on freshwater ectotherms, focusing mostly on aquatic insects. The aim is to improve understanding on the individual variation in thermal strategies and how individuals are able to cope with rising temperature, in particular by studying impacts on feeding and metabolic rates.

Study issue :

The aim of the study was to evaluate the effects of rising temperature on metabolic rates for two aquatic invertebrates : *Aeshna cyanea* and *Limnephilus vittatus*.

What are metabolic rates?

Metabolism is the process in which the body takes and transforms energy to run all biological functions. The speed at which this process moves is termed the metabolic rate.
As temperature sets all biological and chemical reactions, an increase of the temperature will have a strong impact on metabolic rates (Angilletta, 2009).

The influence of changing temperatures on metabolic rates generally follow the same pattern for all animals. Temperature increases metabolism up to a point, the optimal temperature where metabolism is maximized. After this point, when the temperature is too high, the system collapses and leads to the death of the organism (Figure1).

Thermal limits are generally fixed by the temperatures at which the aerobic respiration is not sufficient (e.g lack of oxygen in high temperatures) to cater energetic needs so *oxygen limitation offers the most plausible explanation for the thermal tolerance of organisms* (Angilletta, 2009).

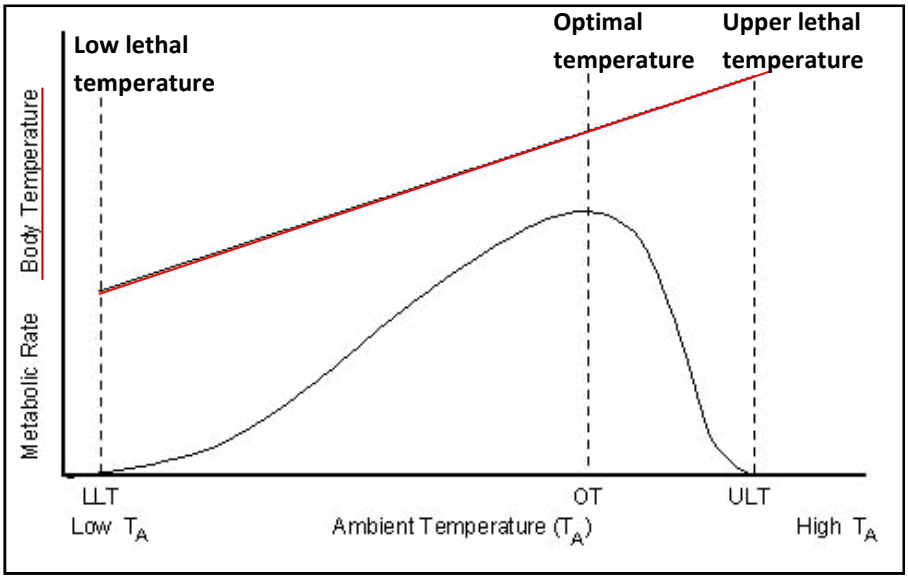


Figure 1 : Theoretic response curve of metabolic rates according to an increasing body temperature
Source : Northern Arizona University

How can we measure metabolic rates?

There are two main ways to measure metabolic rates.
The first and more direct one is the method of calorimetry, which consists in measuring *the rate of heat energy released by an organism*. This method requires precise and expensive instruments so scientists often rather measure the respiration rate, a process that is directly correlated to metabolism. Indeed, the respiration is the process by which animals convert nutrients into energy, which can be used for metabolism (Barnhart, 2017).
For this project, we chose to use respiration rates measurements.

Presentation of the two experiments

- Experiment on *Aeshna cyanea*

Figure 2 : Larva of Aeshna cyanea
Source : R. Thompson

For this experiment, we aimed to measure the Resting Metabolic Rates over **3 different temperatures** (20°C, 24°C and 28°C) and in **two light conditions** (light/dark) to also evaluate the effect of darkness on metabolic rates in *Aeshna cyanea* (Figure 2).

Resting metabolic rates correspond *to the minimal metabolism of an individual in a relatively quiescent state* so we aimed to reduce at minimum metabolic rates by minimizing its activity during measurements.

Aims of the experiment and predictions

AIM	PREDICTION
1) Investigate effects of temperature on resting metabolic rate (RMR) in *Aeshna cyanea* subjected to acute changes in temperature.	• Concerning metabolic rates, we can assume that it's positively influenced by increasing temperature, as a consequence of bodily processes being faster so **mass specific oxygen consumption should be higher in higher temperatures**.
2) Investigate the influence of darkness on metabolic rate.	• As a visual predator, *Aeshna cyanea* should be less active in darkness and consequently the mass specific **oxygen consumption should be lower in darkness**.

- Experiment on *Limnephilus vittatus* (Figure 3)

For this experiment, we used **two groups of larvae** : for the first group (**control group**), we measured metabolic rates (routine metabolic rates) three times at the same temperature (20°C) and for the second group (**temperature treatment group**) we measured metabolic rates through 3 different temperatures.

Activity rate was also measured for both of the group at the same temperature (20°C for the stable group and 16°C, 20°C and 24°C for the other group) to evaluate if activity was affected by temperature in the same way than metabolic rates.

Figure 3 : Larva of Limnephilus vittatus Source : J. Näslund

Aims of the experiment and predictions

AIM	PREDICTION
1) Investigate **effects of temperature on metabolic rates** in *Limnephilus vittatus* subjected to acute changes in temperature.	• As well as *Aeshna* experiment, **mass specific oxygen consumption should be higher in higher temperatures**.
2) Investigate **effects of temperature on activity** in *Limnephilus vittatus* subjected to acute changes in temperature.	• Activity should follow the same pattern in the case of increasing temperature than metabolic rates and **be higher for higher temperature**.

Methods used:

- Metabolic rates measurements

Figure 4 : Respiration rates measurements with the oxygen and temperature sensors
Source : Mathilde Fuentes

In order to obtain the respiration rate of the aquatic insects, we measured oxygen consumed in glass bottles (Figure 4). For this, oxygen concentration was measured two times: a first time to measure the initial quantity of oxygen in the bottle and a second time after 1h in thermal cabinet at the studied temperature. The difference of the two concentrations was then calculated to determine the quantity of oxygen consumed in 1 hour by the individuals.

One oxygen and one temperature sensor recorded data simultaneously. By this method, oxygen consumption could be automatically compensated for temperature. Indeed, the temperature influences the amount of oxygen that can be maximally dissolved in water.
The two sensors were connected to a computer with a software which recorded all the temperature and oxygen consumption data.

- Activity measurement

In order to measure activity of animals (method used only for *Limnephilus vittatus*), we put the individuals in small arenas with a bottom made of sand which were filmed with a camera during 15 minutes.

A tracking software was then used to analyse the video, track the animal's movement and calculate the distance covered in a given time (Figure 5).

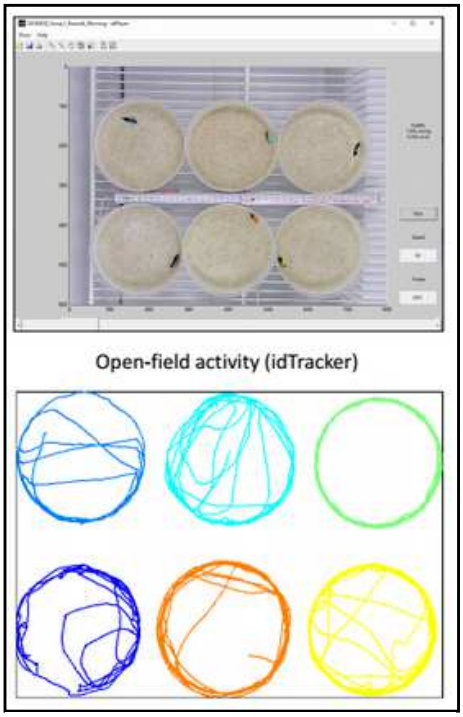


Figure 5 : Open-field activity measurements
Source : J. Näslund

Summary

Introduction

An exponential increase of the average temperature on Earth is ongoing, and by the end of the century the increase is projected to be between 1,8°C and 4°C, as compared to the global average between 1961 and 1990 (IPCC, 2013). This change will have profound effects on Earth's climate, and its ecological systems. The temperature has a major role in ecology and in the evolution of organisms. Most of the living beings are only active in a limited range of temperature and their performance typically follow the same pattern (Sibly et al. 2012).Species differ in their thermal restrictions, and the impact of the temperature is typically more important for ectotherm species (e.g. fish, insects, amphibians) for which the regulation of body temperature depend on their environment (Sibly et al., 2012). Concerning aquatic invertebrates specifically, the body temperature is very close to water temperature so these organisms can hardly deal with change in temperature, contrary to endotherm organisms (e.g. mammals and birds).

Overall, temperature is a significant environmental factor for living organisms because it sets physical limits to all biologic activities. For instance, extreme temperatures will have deleterious effects on enzymes or on the membrane structure of the cells (Somero, 1995), but will also modify the oxygen consumption by the organisms. This oxygen consumption (respiration rate) is directly associated with metabolic rates. Metabolism is the way that body transforms energy to run all its functions. Metabolic rates - the rates which organism take and use the energy - will therefore likely have an influence on growth, reproduction or survivorship (Sibly et al. 2012). As a response to the warming that has occurred over the last 20 years, freshwater animals in general have already increased metabolic rates with 20% (Seebacher et al., 2014).

The aim of this study was to evaluate the acute temperature dependence of metabolic rates of some aquatic insects (caddisfly and dragonfly larvae) across a temperature gradient.

This will give an idea of the acute thermal sensitivity of these insects. The acute thermal sensitivity is defined as a *change in a physiological rate function in response to a rapid change in environmental temperature in the absence of thermal acclimation* (Seebacher et al.,2014).Indeed, it corresponds to short-term reactions of insects to a sudden increase or decrease of temperature. Metabolic rates were chosen to be studied here because this parameter reacts swiftly to changes in temperature (Angilletta, 2009).

The experiments are part of a larger project dealing with responses of predator-prey interactions to increasing water temperatures. This project will enable estimations of how predator energetic efficiency scales with temperature and whether prey mortality and metabolic rates scale similarly or differently with temperature. It is important to understand the role of the temperature on trophic interactions in order to calculate energetic efficiency (individual energy acquisition/individual energy expenditure) which enables definition of the individuals' ability to use resources for growth and, eventually, reproduction (Dell et al., 2014). Indeed, extensive data on energetic efficiency with temperature and body size in multiple freshwater predator-prey systems are currently lacking.

The present experiment constitutes short-term laboratory experiments which contribute as pilot experiments to obtain baseline comparative data for temperature responses to be used in future major experiment. Indeed, such initial investigations are of major importance for the efficient progress of research projects since they lead to better experimental design large-scale projects.

The first part of this report will briefly present the University of South Bohemia and the biology laboratory which conducts this study. The second part will talk about the methods and the material used in this project. The results will be presented in a third part and finally discussed in the last part of this report.

I. Presentation of the internship's place and context of the study

Jihočeskà Univerzita (JU) is located in the region of South Bohemia, in the southern part of the Czech Republic in the city of České Budějovice (Figure 6).

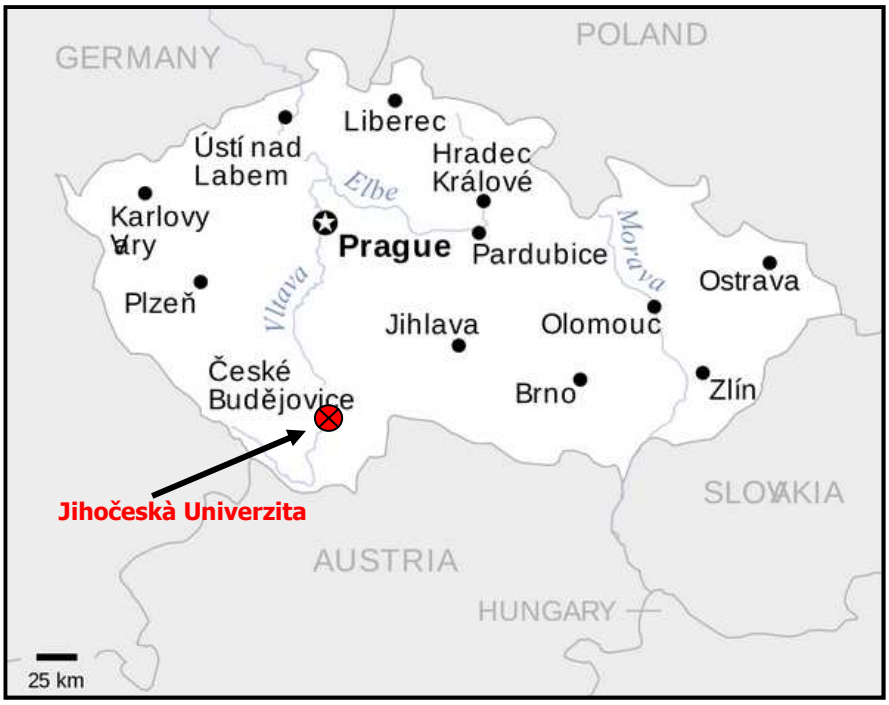


Figure 6 : Location of Jihočeskà univerzita (University of South Bohemia in České Budějovice)
Source : http://czechrepublicmap.facts.co

This university was created in 1991 and consists of 8 faculties, including the Faculty of Science and the Faculty of Fisheries and Protection of Waters. The university shares its localities with the Czech Academy of Science (CAS). I did my internship within a research group (The Aquatic Ecology Lab) which is split between the Department of Ecosystem Biology at the Faculty of Science and the Institute of Entomology at CAS.

The Aquatic Ecology Lab mainly conducts studies on aquatic ecology, especially on evolutionary ecology and population dynamics of predatory freshwater insects and fish. Ongoing projects (2017-2019) concern the role of phenotypic plasticity in life histories and trophic interactions under anticipated climate change.

The aim of this study was to run short-term laboratory experiments to bring baseline information on thermal response concerning two aquatic insects. This information will be used for future larger scale experiment concerning the way that aquatic insects reacts are able to cope with climate change.

II. Materials and Method

In order to address the question of how metabolic rates scale with changes in temperature, two different experiments were conducted. One of them investigated larvae of dragonflies (*Aeshna cyanea*; see Box 1) and the other one investigated larval caddisflies (*Limnephilus vittatus*; see Box 2).

II.1. Aeshna experiment

We chose to carry out experiments on *Aeshna* to evaluate both temperature and light effects on resting metabolic rates. Resting metabolic rates (RMR) correspond to *minimal metabolism of an individual in a relatively quiescent state* but it can constitute up to 50% of energy expenditure for one individual(Burton et al., 2011).This kind of metabolic rates is generally constant because individuals seem to be unable to reduce RMR even during periods of intense energy expenditure (Burton et al., 2011).

II.1.1. Procedure

II.1.1.a- Capture of individuals

All invertebrates used during the study were captured in wetlands and ponds in the vicinity of České Budějovice, Czech Republic. Aeshna were collected in the area of Řídká Blana from the bottom sediment of small forest ponds (Figure 7).

Figure 7 : Forest ponds in Řídká Blana where individuals of Aeshna cyanea were collected
Source :T. Křeček and Boza paja

After capture, they were brought to the laboratory where they were housed individually isolated in plastic containers for the duration of the study, to avoid cannibalism and to eliminate any potential effects of interindividual interactions. Each container was labelled with the date of collection and a specific ID-number.

The *Aeshna* larvae were left in a temperature regulated room (20°C) with a photo period of 16h (light) : 8h (dark)and fed each day with chironomids.

BOX 1: Focus on *Aeshna cyanea*

Aeshnidae is the dragonfly family which contains the highest number of species in Europe and *Aeshna cyanea*, also commonly named the Southern Hawker, is one of the most common species of the genus in Central Europe (Boudot, 2014).

Larvae of *Aeshna cyanea* are present in shaded standing waters, such as small forest ponds but also in slow-flowing rivers and oxbow-lakes, with a preference for leaf litter substrates. The larvae often reside in coarse debris (wood, leaf) where they ambush other aquatic invertebrates, tadpoles and even small fishes. The larvae will emerge into the adult stage after 10 or 11 molts (two years) (Tachet et al., 2000).

The larva (Figure 8) can be distinguished by its elongated body shape and the shape of its long labial mask (3,5 times longer than broad : see Figure 9). The lateral spines along segments 6-9 are also good characteristics to determine this species (Bowles, 2010).

Figure 8 : Aeshna cyanea larva.
Source : A. Karwath

Figure 9 : Aeshna cyanea's mask
Source : L. Pelissier

Concerning adults, they are readily recognizable by a black body with blue and green spots (for the male : Figure 10) and a brown body with green spots (for the female) (Bowles, 2010).

Figure 10 : Aeshna cyanea adult (male)
Source : D. Heidrich

II.1.1.b- Experiment

The aim of this experiment was to measure resting metabolic rates of Aeshnas across 3 temperatures (20°C, 24°C and 28°C).

Resting metabolic rates are *minimal rates of energy metabolism* (Burton et al., 2011). Therefore we aimed at reducing the metabolic rates to a minimum in the experiment. First of all, before measurements, the Aeshna were starved 24h so that the digestion would not affect metabolic rates. Secondly, shelter-structures were put in the metabolism chambers (bottles) in order to reduce the activity of the larvae (Näslund & Boukal, 2017). The shelter-structures were made of small bent aluminium grille, allowing the Aeshna to either hide inside or to climb it.
Finally, two light-conditions were applied. Half of the respirometry bottles were covered with aluminium foil to see if there is any effect of darkness on metabolic rates whilethe other half acted as control treatment, with bottles not being covered (Figure 11).

Figure 11 : The 2 light-treatments
Source : J. Näslund

The trial day, Aeshna larvae were put in glass bottles (270mL) filled with distilled water with a magnet and a shelter and left in thermostatic cabinet (Lovibond©) during half an hour at 20, 24 and 28 degrees.

For each temperature there were 4 control bottles (2 light and 2 dark) without animals inside to control for potential background consumption of oxygen by bacteria or algae in the bottles.

After half an hour of acclimation (applied to let the larvae settle after the introduction to the bottle), the oxygen consumption was measured in each bottle with oxygen and temperature sensors(starting with the 20°C treatment; finishing with the 28°C treatment) during 10 seconds (Figure 12). Two optodes (Unisense©) took simultaneous measurements and oxygen concentration was automatically compensated according to temperature. Indeed, oxygen consumption needs to be compensated because less oxygen can be dissolved in higher temperatures so it needs to be taken into account. The oxygen sensor has a high level of detection that allowed detecting very small changes in concentration inside the bottles.

Both sensors were connected to a software(Sensor Trace©)which took one measurement of temperature and oxygen per second (and recalculated the measurements compensating for temperature) and enabled to see in real time the concentrations measured (draws the curve while measuring).

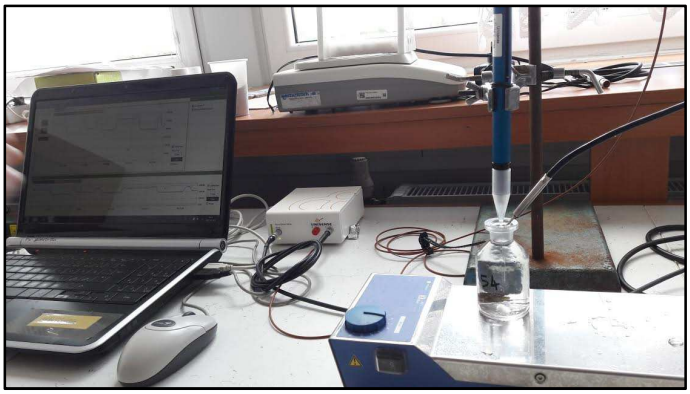


Figure 12 : Measure of oxygen consumption with the temperature and the oxygen sensors connected to the computer
Source : M. Fuentes

After the measure, bottles were placed in the thermostatic cabinet for another 1 hour. Finally, oxygen consumption was measured again and after the experiment, *Aeshnas* were weighed (wet mass) in order to calculate later, the mass specific consumption of each individual.

All in all, 99 *Aeshna* larvae were tested and each individual experienced only one temperature and one light condition. One of these individuals was excluded from analyses because it was the only individual within instar F-2 (all other individuals were F-1 or F-0). All the steps of the experiment are summarized in Table 1.

Table 1 : All the steps of the experiment. The arrow indicates progress in time.

Dragonflies put in bottles after 24h of starvation
Half of the bottles covered with aluminum foil
30 minutes in thermal cabinet (acclimation) at 20°C, 24°C or 28°C
Measure of Metabolic Rates (20°C→24°C→28°C)
1h in thermal cabinet
Measure of Metabolic Rates (28°C→24°C→20°C)
Weighing of the individuals

II.2- Trichoptera experiment

We chose to carry out experiments on *Limnephilus vittatus* (see Box 2) to evaluate effects of temperature on metabolic rates.

BOX 2 : Focus on *Limnephilus vittatus*

Limnephilus vittatus is a caddisfly which belongs to the family of Limnephilidae. This family name comes from *Limne*, meaning marsh or lake in greek, so the species is typically associated with ponds, lakes or even low streams. Limnephilidae species are case-bearing caddisflies and larvae of *Limnephilus vittatus* build a case made of small sand grains (Wiggins, 2004).

The larvae are shredders, consume algae and aquatic plants (Figure 13).

Figure 13 : Two larvae of *Limnephilus vittatus* with and without case
Source : A. Chalkley

Adults are nocturnal insects with have brown wings and long antennas (Figure 14). They can be mixed up with nocturnal moths, but differ from these by having hairs and not scales on their wings (Amateur Entomologists' Society, 2017).

Figure 14 : Adult *Limnephilus vittatus*
Source : Shane58

II.2.1- Procedure

II.2.1.a- Capture of the individuals

Caddisflies were collected near Jindřichův Hradecin, in a shallow pond (former fish pond) rich in vegetation and the majority of the individuals were found, fixed to aquatic vegetation (*Sparganium sp.* Figure 15).

Figure 15 : Sparganium plant in a pond
Source : B. Kimball

Caddisflies were brought to the laboratory and housed individually in individually labelled plastic cups with strands of *Sparganium* for the duration of the study.

All in all, 64 individuals were trialled, separated into 2 treatment groups.

II.2.1.b- Experiment

Metabolic rates measurements

-First group of individuals

For the first group (48 individuals), 3 different temperatures were tested (16°C, 20°C and 24°C).The trials days, caddisflies were put in glass chambers (≈4mL) with a magnet and a small grid at the bottom so that they can fix at it (Figure 16). These chambers were filled with distilled water at right temperature (16°C, 20°C, or 24°C) and left in thermostatic cabinet (Lovibond©) few minutes for acclimation time before measurements. Concerning the measurements, the method was the same as for Aeshna, with ≈1h between 2 measurements, using oxygen and temperature sensors to measure oxygen concentration, from which we could calculate oxygen consumption rates.

Figure 16 : Glass chambers used in the experiment

-Second group of individuals

The second group of individuals (16 individuals) acted as a "control group" (controlling for effects of trial-order, i.e. whether the first trial elicit stronger responses than the following trials) and metabolic rates were measured three times at a stable temperature of 20°C (following the same trial protocol than the first group).

After each day of metabolic rates measurements, all the individuals were weighed with their case. After the third round of measurement, we weighed the animals without their case and the case itself alone. Based on these measurements, we could subtract the weight of the case from the previous measurements.

Activity

The day after each metabolic rate measurement, the insects were used for an activity experiment.

The individuals were put in round arenas with a bottom made of sand (Figure 17a) so they can easily walk on it, and filled with water at the right temperature. Arenas were placed inside the Lovibond (Figure 17b) and a camera, at the top of the arenas, filmed the arenas during 15 minutes. Two fluorescent tubes were also placed on the walls of the Lovibond to light the arenas and obtain a sufficiently good video quality.

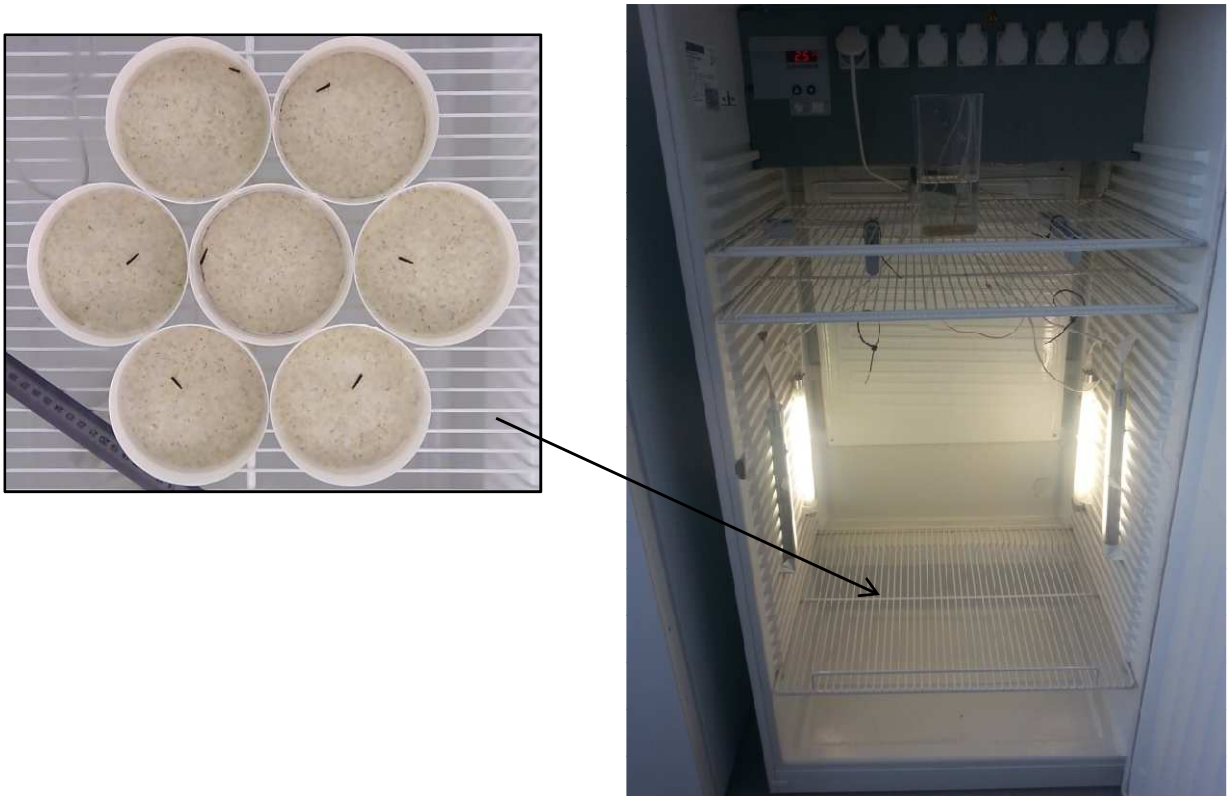


Figure 17 : Activity measurements
Figure 17a : Arenas with the bottom made of sand with caddisflies inside (the ruler at bottom-left serves as a reference at the start of the recording, when it is placed in the centre of the image)
Figure 17b : Interior of a thermal cabinet where recording of activity was made

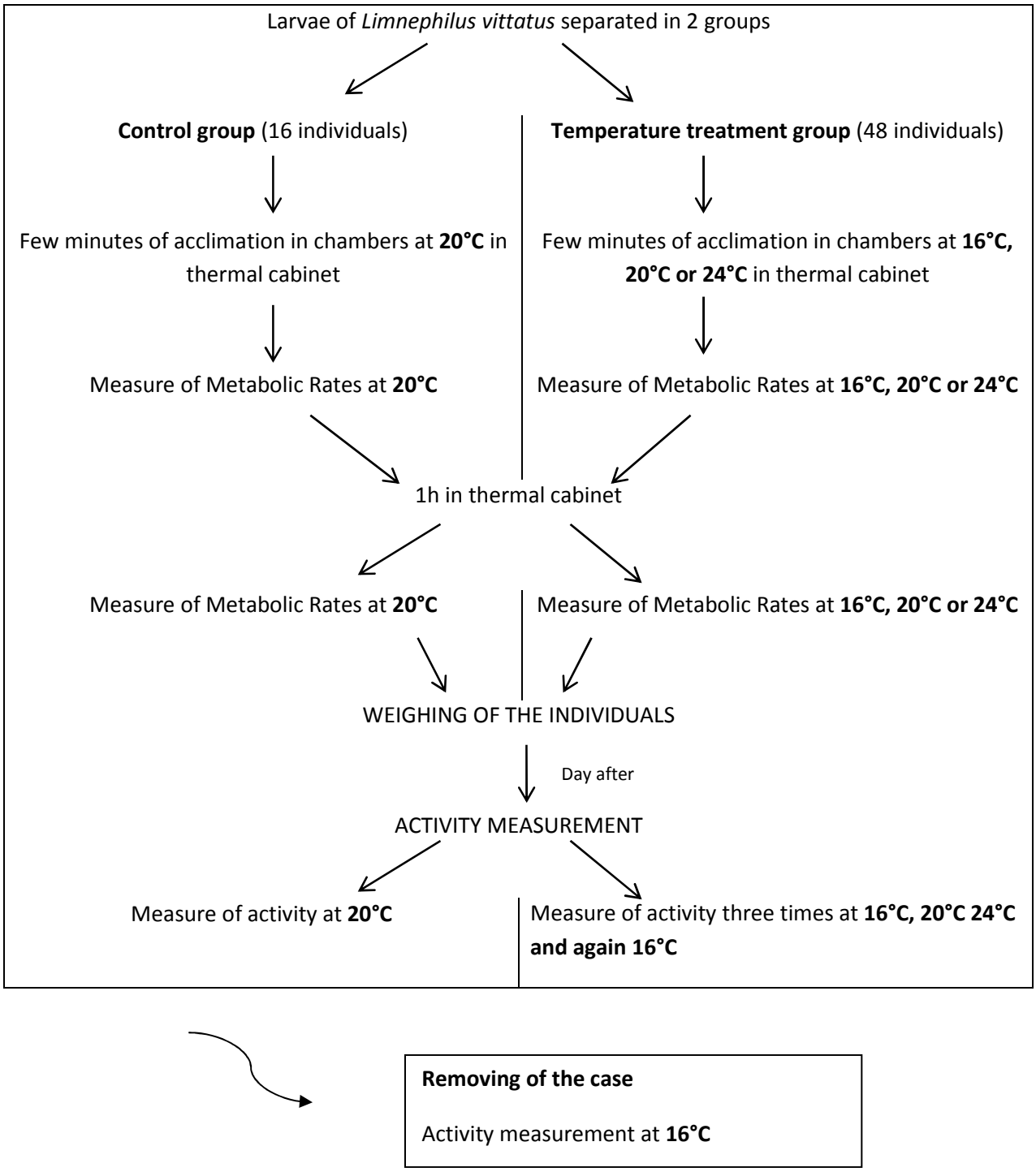
For the treatment group, activity was measured at 16°C, 20°C, 24°C and then, again at 16°C and for the stable group, it was measured three times at 20°C, like metabolic rates.

Additional activity measurements

At the end of the experiment, the cases of all the individuals were removed (individuals were pushed away from their case with the blunt end of a needle) and they were left in cups with sand so that they could build a new one. Activity rates were measured afterwards to investigate how forced case-construction affected activity. This part of the experiment was not associated with the original aims, but the data nevertheless provide useful information for the interpretation of the other data.

All the steps of the trichoptera experiment are sum up in the Table 2.

Table 2 : Trichoptera experiment resumed with all the steps



II.3. Calculations

II.3.1- Metabolic rates

For both experiments, oxygen consumption rate was calculated based on measurements of oxygen concentration. It was calculated as the difference between the first oxygen measurement and the second one (after ≈1h) (given in µL/L), which was multiplied with the water volume of each respiration chamber and then divided by the weight of each individual (without the case for caddisflies).Finally values were divided by the exact duration between 2 measurements in hours to obtain the mass specific oxygen consumption in µL/g/h. The change in oxygen concentration, as measured in the control bottles (bottles without any individuals inside), was added to the measurements for the animals to correct the data for background oxygen depletion.

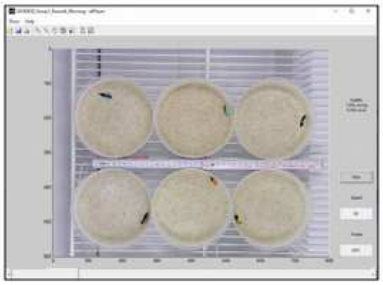
II.3.2- Activity

In order to analyze activity measurements that were done, idTracker (Pérez-Escudero et al., 2014) was used. This tracking software is suitable for tracking animal's motion.

This kind of software relies on 3 main steps :

1. Background removal (all non-moving parts of the video are eliminated).
2. Individual detection (based on contrast differences).
3. Tracking of the animal through each frame of the video.

When there are several individuals on the same video file (it was the case with caddisflies), the software is capable to analyze movements of all animals in the same time while maintaining the correct identity for each one (Pérez-Escudero et al., 2014).



Open-field activity (idTracker)

At the end of the tracking, the software draws the pattern of the movements made by each individual (Figure 18) and the distance covered can be calculated based on the Euclidean distance between coordinates from consecutive frames of the video.

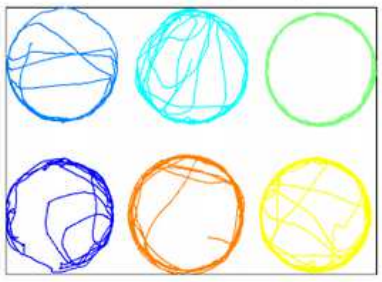


Figure 18 : Exemple of insect tracking
Source :J. Näslund

II.3.3- Statistical analyses

The statistics software R was used to analyse the data and build models to interpret it.

The trichoptera experiment was based on repeated measurements and each individual experienced 3 different temperatures over time (data for each individual need to be considered as non-independent to avoid issues of pseudo replication). So individuals were statistically non-independent contrary to the Aeshna experiment where each individual experienced one temperature.

Normality of the data was evaluated based on residual plots, and found to be acceptable for linear modeling.

Concerning the trichoptera experiment, it is possible that there is an "individual effect", i.e. some consistent variability among individuals. To take this potential individual consistency into account, we chose to construct a model that considers each individual as a "random factor" (contrary to the temperature, which is a fixed factor because we picked the values intentionally).

In order to analyse our data with a random factor included, we used a linear mixed model, which is a statistical method which can handle both fixed and random variables (particularly useful when repeated measurements are made). It corresponds to the function lme (lme: linear mixed effects) in the R-package nlme (linear and non-linear mixed-effects models) (Pinheiro and Bates 2017). For the caddisfly experiment, an example of a formula that we used is specified below.

*lme(log(M.S.M.O2) ~ log(Mass) * Day,random=~1|ID)*

With this formula we tried to represent the mass specific consumption (M.S.M.O2, log-transformed) depending on the mass of the individuals (log-transformed)and the day of the experimental trial (here day can be replaced by temperature treatment because a day of experiment corresponded to a specific temperature) by using a linear mixed model (with individual as a random factor : "random=~1|ID").

For the Aeshna experiment, we used a simple linear model (function lm in R):

lm(log(M.S.M.O2) ~ Instar +Light.TR + Sex +Shelter.Index +TR

This formula was used to represent the log of the mass specific oxygen consumption (M.S.M.02) depending on the instar, the light treatment, the sex, the shelter index and the temperature treatment (TR). Before each respiration measurements and after individuals were come out of the Lovibond, we checked if the Aeshnas used the shelter or not, to construct the shelter index . If they didn't use it at all, we assigned a value of 0, if they use it once before the first or second measurement, we assigned a value of 1 and if the individual used it both times we checked it, then we assigned a value of 2.

Significance of the factors included in the linear models was assessed based on analysis of variance, with Type III sums of squares.

III. Results

III-1. Trichoptera's experiment

III-1.1 Metabolic rates

III.1.1.a- Temperature treatment group

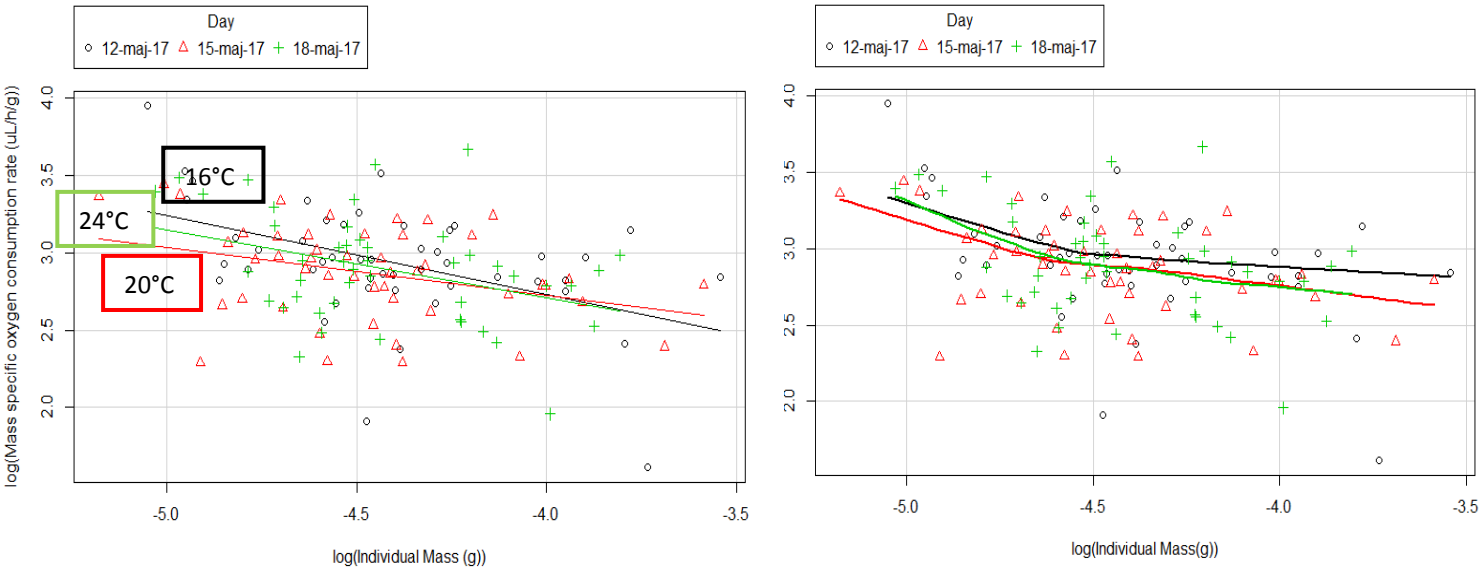


Figure 19 : Graphs representing the log(Mass Specific Oxygen Consumption) function of log(Mass).
Figure 19a : Linear regression
Figure 19b : Curve with loess smoother (i.e. : local regression)

The two graphs above (Figure 19) represent the mass specific oxygen consumption depending on body mass and day (each day corresponds to a specific temperature). The graph on the left (Figure 19a) is based on a linear model whereas the other graph is a curve with loess smoothers (i.e. local regression, Figure 19b).

If we focus on the general trend, the oxygen consumption seems dependent on the individual mass and the local regression curve suggest a mass effect more important for smaller individuals. Nevertheless, the effect seen here is likely based on a non-linear size-metabolism relationship (with the loess smoother, the slope is more important before a value of -4.5 in log (individual mass), and after this value the effect of mass appears to decrease; see Figure 19b). Therefore, despite the significance of this variable in the Anova test (Table 3), we can assume that the general size-metabolism relationship is relatively weak within the investigate size-span.

Table 3 : Summary of the statistical test of significance for factors included in the linear mixed model of oxygen consumption in the *Limnephilus* experiment for the treatment group.

```
> Anova(lmm1.met,type="III")
Analysis of Deviance Table (Type III tests)

Response: log(Mass.Spec.M.O2)
                         Chisq Df Pr(>Chisq)
(Intercept)             1.5190  1   0.217773
log(Corr.Ind.Mass)     14.2157  1   0.000163 ***
Day                     1.2926  2   0.523985
log(Corr.Ind.Mass):Day  1.5586  2   0.458737
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Concerning the effect of temperature on oxygen consumption (factor: Day), it is not significant in the applied model. Indeed, the 3 curves are relatively similar (Figure 19; more visible in the local regression model, Figure 19b) and the ANOVA test (Table 3) confirms the absence of significant difference in oxygen consumption through the 3 temperatures.

III.1.1.b- Control group

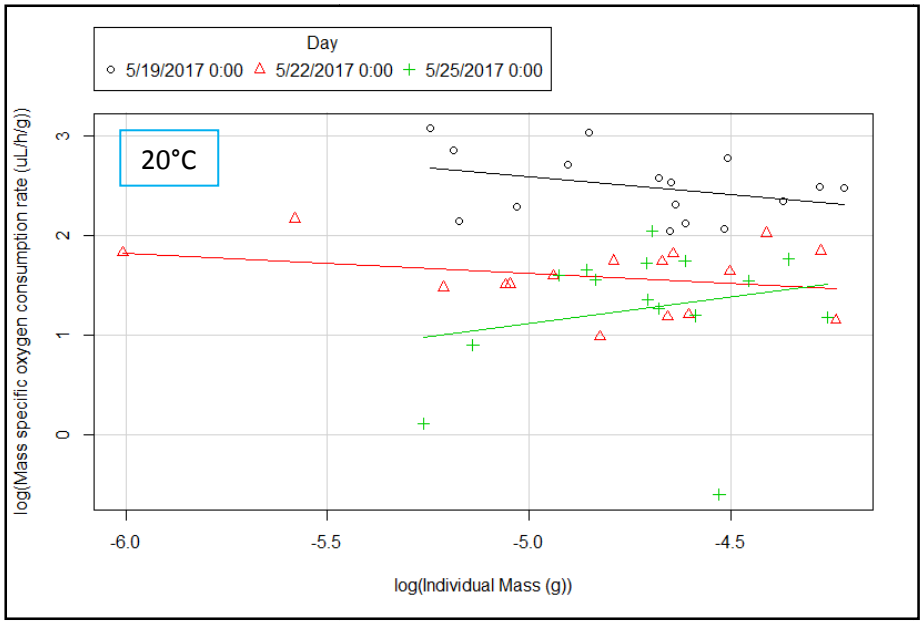


Figure 20 : Linear regression of the log of the Mass specific oxygen consumption function of the log of the individual mass for the control group

The same representation/model was chosen for the stable group (Figure 20) and the results are noticeably different. Here, the day (i.e. the order of the trials) appears to have an impact on oxygen consumption despite it being measured at the same temperature each time.

The first day of measurements (black line), the caddisflies show higher oxygen consumption, compared to the second and the third day of measurements (which are quite similar to each other: red and green points are located in the same general area of the graph (Figure 20).

The significance of the factor Day is confirmed by the Anova test (Table 4). No strong indications of effects of body mass were found (see Figure 20; Table 4).

Table 4 : Summary of the statistical test of significance for factors included in the linear mixed model of oxygen consumption in the *Limnephilus* experiment for the control group.

```
Response: log(Mass.Spec.M.O2)
                     Chisq Df Pr(>Chisq)
(Intercept)         3.1222  1    0.07723 .
log(Corr.Ind.Mass)  0.4575  1    0.49879
Day                66.4009  2  3.813e-15 ***
```

III.1.2 Activity

III.1.2.a- Temperature treatment group

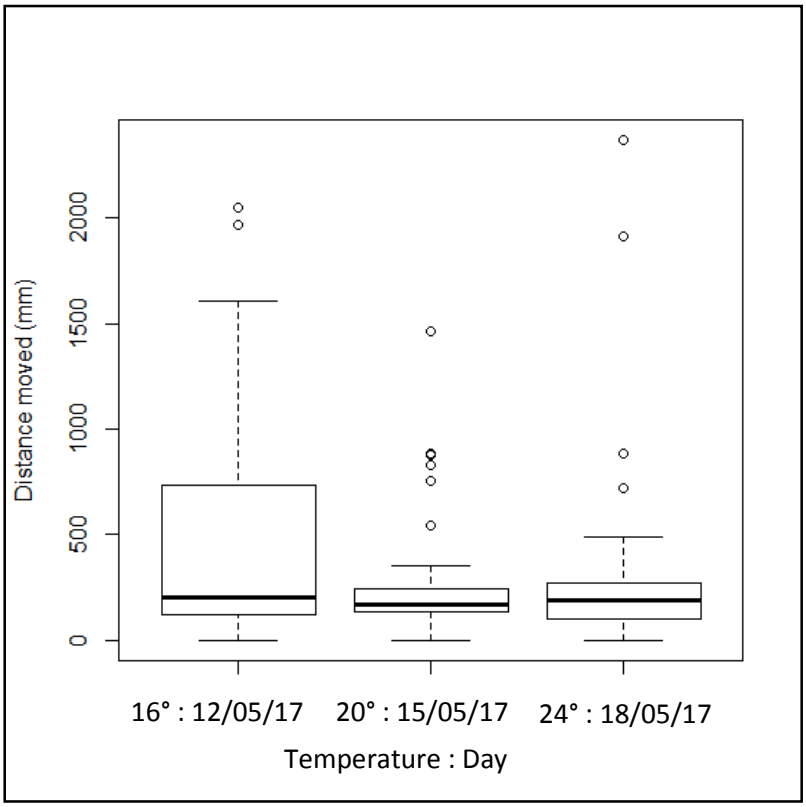


Figure 21 : Boxplot of the distance moved in function of the temperature for the treatment group

The graph of the activity depending on different temperature (Figure 21) does not show strong differences through the 3 days of measurements and the three different temperatures. The median values are quite similar but the first day of measurements (16°C) depicts more variability: the values are higher and more spread than for the two others temperature.

III.1.2.b- Stable group

Concerning the stable group (Figure 22), the 3 boxplots are relatively similar but for the first day of measurements, activity seems to be slightly higher (even if it is not significant if we look at the spread of the values).
At the second day of measurements, the values appear to tighten around the median value and there is less variability in the distance moved.

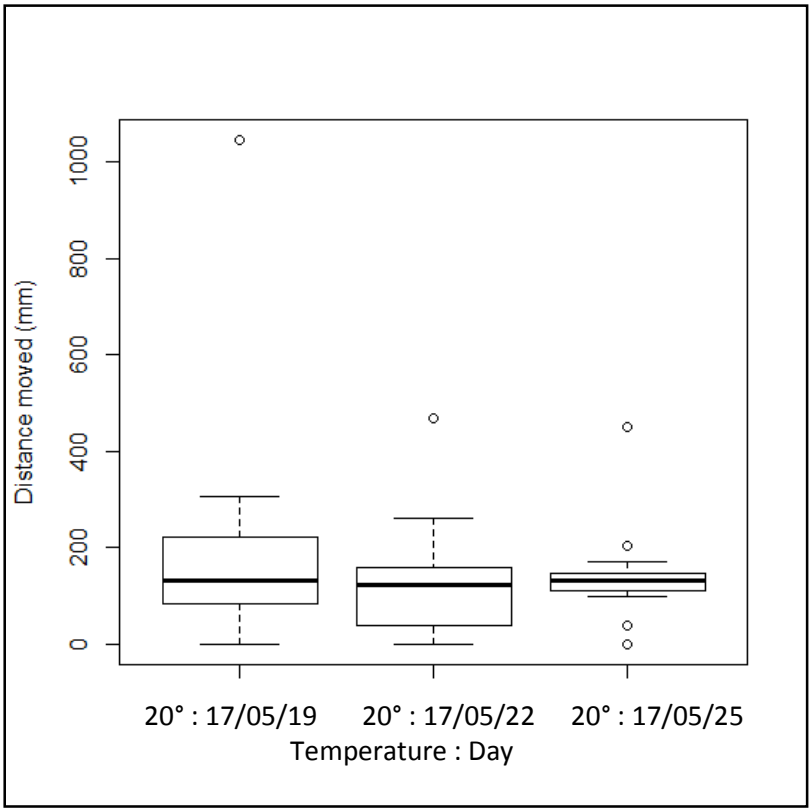


Figure 22 : Boxplot of the distance moved in function of the temperature for the control group

III.1.2.c- Overall activity

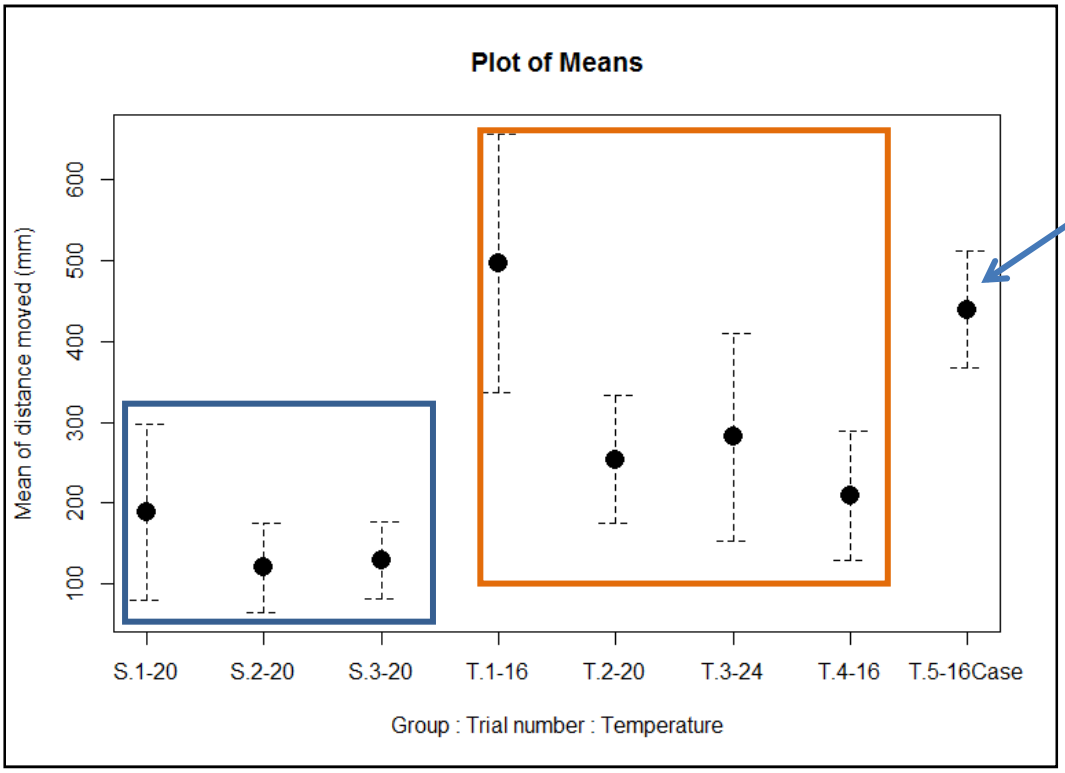


Figure 23 : Overall activity
The stable group is marked with a blue line and the treatment group with a orange line
The last point on the right of the graph corresponds to the "extra experiment" we made by removing the original case of the individuals and measuring activity afterwards.
The picture on the side shows 2 larvae : one with a new building case (the lightest color) and the other one with an original case.

The graph above (Figure 23) represents the overall activity, across all treatments and including the extra trials following the main experiment.

We can observe that the first day of measurements depicts always a higher average activity). It is particularly visible for the treatment group because the first measurement at 16°C corresponds to a mean activity of ≈500 and the second measurement at 16°C is surprisingly almost halved. The second and the third measurement (20°C and 24°C) are between 200 and 300 whereas the activity rate should be higher than at 16°C with the increasing temperature, if our initial prediction were true.

The last point shows a relatively high activity by caddisflies despite the fact that they spent a lot of energy by building a new case. This shows that the larvae still have a capacity for high activity, and were not exhausted or diseased as the experiment progressed.

III.2- Aeshna experiment

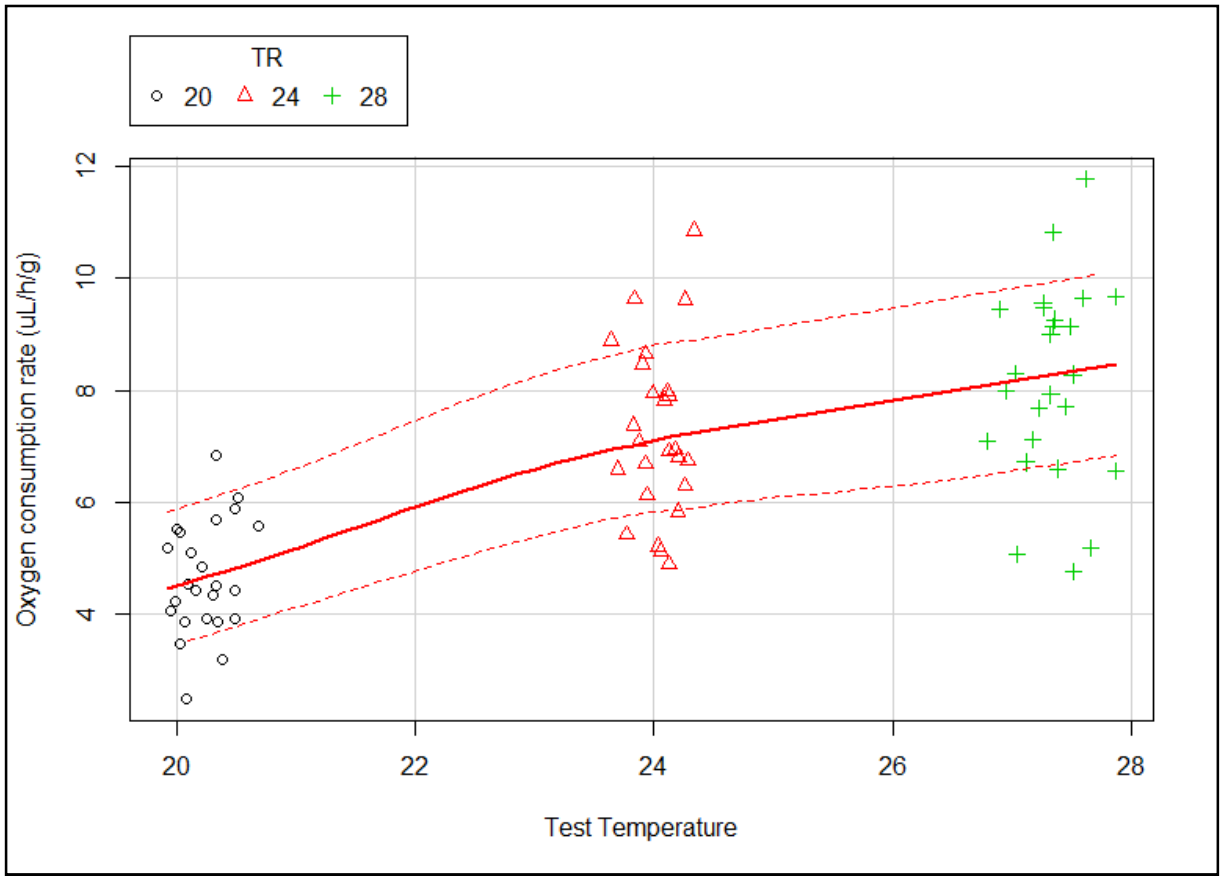


Figure 24 : Oxygen consumption rate according to different temperatures for *Aeshna cyanea*

If we look at the result concerning *Aeshna* experiment (Figure 24; Table 5), we can see that the temperature significantly impacts the quantity of oxygen consumed by the Aeshnas.

The consumption is lowest at 20°C and then, increases with increasing temperature. We can notice that the curve looks like a theoretic temperature response curve (Figure 25): the slope is more important at the beginning (between 20°C and 24°C) and dampened when the temperature is increasing from 24°C to 28°C. After 28°C, the oxygen consumption will probably stabilize to reach a plateau until the curve rapidly decreases toward zero when the temperature becomes too hot to allow for the survival of the *Aeshna* larva.

Table 5: Summary of the statistical test of significance for factors included in the linear model of oxygen consumption in the *Aeshna* experiment.
- "Light. TR" means light treatment (light or dark)
- "TR" is for the temperature treatment.

```
Anova Table (Type III tests)

Response: log(M.S.M.O2)
               Sum Sq Df  F value     Pr(>F)
(Intercept)   16.8788  1 334.2169 < 2.2e-16 ***
Instar         0.0480  1   0.9514     0.3329
Light.TR       0.0022  1   0.0442     0.8341
Sex            0.0013  1   0.0265     0.8711
Shelter.Index  0.0381  1   0.7553     0.3879
TR             4.0625  2  40.2206 3.348e-12 ***
Residuals      3.3837 67
```

The Anova test above (Table 5) shows all the parameters of the experiment and their influence (or not) on oxygen consumption by aeshnas. This table suggests that the temperature is the only parameter which affects the oxygen consumption of the Aeshnas.

The light treatment and the instar of the larvae have apparently no effect either on oxygen consumption.

IV. Discussion

IV.1. Aeshna experiment

IV.1.1. Effect of increasing temperature on resting metabolic rates

According to the Metabolic Theory of Ecology (MTE) in most cases, metabolic rates of insects increases with the temperature (Sibly et al., 2012) and this trend is confirmed in the results of Aeshna experiment.

The range of temperatures studied corresponds probably to the part of the thermal response just before the optimum of metabolic rates because the slope elevation tends to decrease and will apparently stabilize at an average value between 9 and 10µL/h/g (Figure 25). Further experiments at higher temperatures than 28°C should allow finding the optimum temperature (for which metabolic rates is the highest), and after which the conditions would have deleterious effects on the animals.

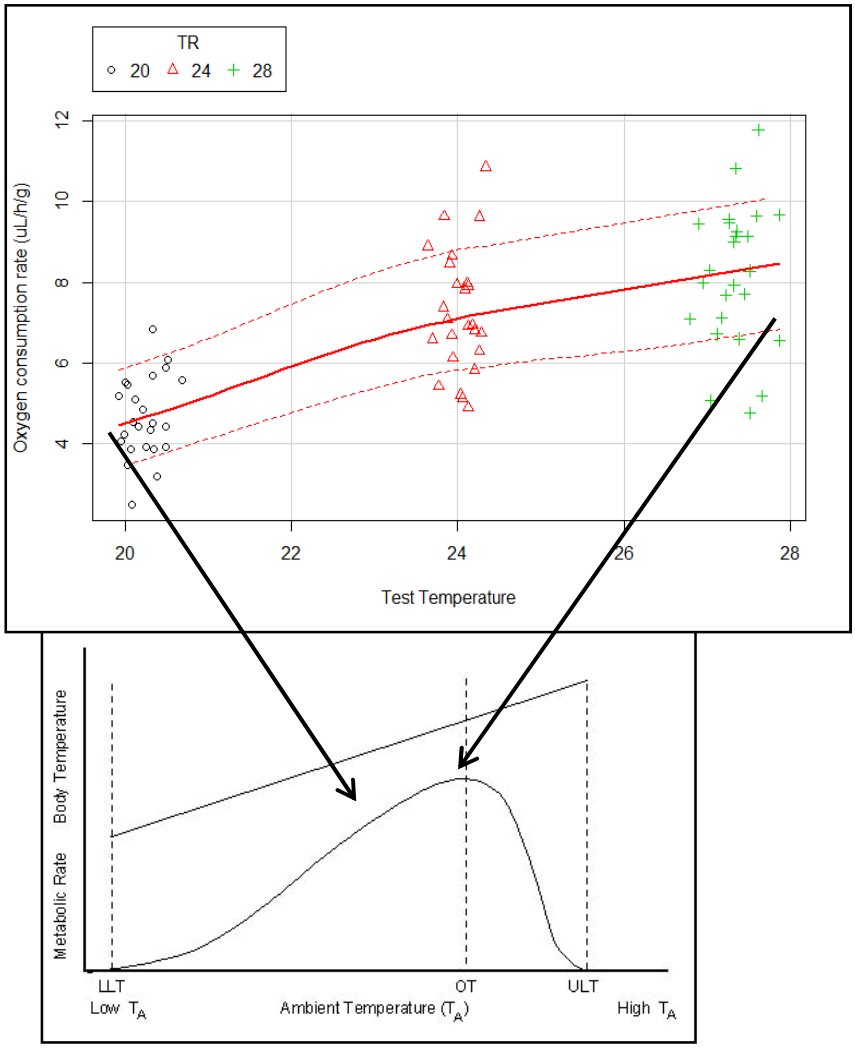


Figure 25 : Correspondance between the experimental response to temperature in Aeshna and the theoric thermal response curve

Consequences of higher resting metabolic rates

There are several assumptions concerning high resting metabolic rates in the scientific literature but Burton et al. (2011) talk about two opposite hypotheses concerning benefits for individuals by having low or high resting metabolic rates:

- The "*compensation hypothesis*": individuals with a lower RMR would have a better fitness because of a lower self maintenance (they can use more energy in growth or reproduction).

- The "*increase intake hypothesis*": individuals with a higher RMR would have a better fitness because of larger internal organs and higher maximum metabolic rates resulting in a greater assimilation of the energy for growth or reproduction. Moreover, individuals with a higher RMR tend to be more aggressive and dominant towards the others so they have an easier access to food.

However, these two hypotheses can be balanced and high RMR, as well as low RMR can be a benefit or a disadvantage according to the environmental conditions.
On the one hand, in optimum conditions, where food is accessible and abundant, a higher RMR would be an advantage because of the easier access to food and a better capacity for growth. On the other hand, in more unfavorable environments, individuals having a low RMR would be more resilient because of low maintenance requirements.

IV.1.2- Effects of the others parameters

The others parameters of the experiment apparently do not markedly affect resting metabolic rates. It is quite surprising that the light or the darkness don't show any difference in oxygen consumption by Aeshnas.
Indeed, we know that dragonflies are diurnal organisms and their activities are generally lower at night, preferring to roost in trees or plants until morning (Hong-Qiang, 2006) but reports mentioning metabolic rates of dragonflies at night are relatively scarce. It would be interesting to conduct further research on it. For instance, it might be possible that measurements in darkness during night-time show different results, if the larvae are affected by strong dial cycles in their metabolism. A high day-metabolism might not be affected by darkness alone.

Previous studies on Aeshna in the lab, has shown significant effects of sex and the use of shelters (Näslund & Boukal, 2017), but this doesn't appear to be the case here. Sex effects could possibly be variable depending on season or life-stage. The fact that shelter had no effect is an indication that the standardization of activity actually worked, which means that the shelter design we used could be reused for future experiments without add bias in the results. The previous experiment where shelter effects were found utilized smaller shelter-structures, which could have allowed for more variable utilization of the shelter-structure.

Concerning instars, two late intars of Aeshnas were used in the analyses (F-0 and F-1; a single F-2 individual was excluded from the analyses). This factor did not impose differences in mass specific respiration rates. However, based on the indications of size effects from Figure 19b, we can assume that results would have looked different if smaller instars had been included. Indeed negative allometric scaling of metabolism is common in animals (Sibly et al., 2012).

IV.2. Trichoptera experiment

IV.2.1- Effects of increasing temperatures on metabolic rates in *Limnephilus vittatus*

Contrary to Aeshna results, the trichoptera experiment did not show the expected pattern of increasing metabolism with increasing temperature. Indeed, there was no significant difference in metabolic rates through the different temperatures.
The control group helped to understand this phenomenon because metabolic rates were relatively high the first time of measurements and decreased for the two following measurements. Thus, it looks as if individuals were stressed the first time they experience a new experimental condition (i.e. they consume more oxygen). After the first experience, however, it seems like they remember or recognize the situation and adapt their behavior to it, which reduce their respiration rate. Therefore, at first sight, and if we focus only on the temperature treatment group, we cannot see the temperature effect since it might be masked by this "experience effect".
Indeed, respiration rates at 16°C could very likely be lower than at 20° or 24° but because of this experience effect, it is not detectable in the repeated measurements. This effect is very important to consider in follow up experiments on trichoptera larvae.

Similar effects are also visible in the activity results with the two measurements at 16°C (trial 1 and trial 4 Figure 23) which show a significant difference in their activity rates in the same temperature. Furthermore, the last activity trial (following rebuilding of the case; i.e. trial 5) shows that the activity is not limited at later trials, since it increased dramatically between trial 4 and trial 5. The average of the distance moved at trial 5 in 16°C is well above the measurements at 20°C and 24°C.

IV.2.2. Consequences of forced-building case on activity

For caddisflies, the case brings protection against predators but involves also a lot of energetic expenditures because of the building and carrying of those cases (Correa-Araneda et al., 2017). Consequently, we could have expected activity rates to be lower after building the new case than with the old one, but this effect is not observed in the our experiment.

Two possible approaches may be envisaged:
- Caddisflies lost their experiential references after the removing of their cases and were stressed again by experiments.
- Caddisflies spent a lot of energy on building a new case, so they need to be more active to find food and regain the lost energy.

Precaution should be taken concerning the conclusions we can draw from the results, because many biotic and abiotic parameters existing in nature can play a role in how the insect cope with changes in temperature. Those parameters cannot be all reproduced in laboratory so we can't ascertain that the response curve we obtained through experiments is the same than in real conditions.

Conclusion

This study provided results that can be used to investigate and better understand the reactions and adaptations in the metabolic rate of aquatic invertebrates, when facing increasing temperatures.

Indeed, in the case of Aeshna experiment, the results were what we expected, with an increasing of resting metabolic rates with rising temperatures. The temperature for peak resting metabolic rates should likely be around 30°C, meaning that a temperature higher than that could have deleterious effects on this species in the long-term.
This experiment with dragonfly larvae also allowed discovering that darkness apparently did not affect metabolism, as indicated by respiration rate.

Concerning the experiment with a trichoptera species, *Limnephilus vittatus*, it highlighted a phenomenon that has hitherto not been mentioned in scientific reports for this kind of species, which is an “experience effect” (or “learning effect”). The larvae are potentially able to remember an experience and adapt when it copes with this experience again. That is likely what happened in the repeated respiration trials, which led to temperatures being masked.

Overall, the two experiments resulted in new insights into the methodology of laboratory temperature-response experiments and also some useful data that can be used when planning future studies on this subject. The results also raise several new questions that could open up new opportunities of research for the laboratory.

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