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Thermal tolerance of two mound-building wood ants *Formica aquilonia* and *Formica polyctena* and their hybrids

Master's thesis

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May 2020

Tiedekunta – Fakultet – Faculty

Bio- ja ympäristötieteellinen tiedekunta

Tekijä – Författare – Author

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Työn nimi – Arbetets titel – Title

Formica polyctena ja *Formica aquilonia* kekomuurahaisten ja niiden risteymien lämpötilakestävyys

Oppiaine/Opintosuunta – Läroämne/Studieinriktning – Subject/Study track

Ekologian ja evoluutiobiologian maisteriohjelma

Työn laji – Arbetets art – Level	**Aika – Datum – Month and year**	**Sivumäärä – Sidoantal – Number of pages**
Maisterintutkielma	Toukokuu 2020	51 + liitteet

Tiivistelmä – Referat – Abstract

Maapallon keskilämpötila on jatkuvassa nousussa. Populaatiot voivat sopeutua muutokseen, jos niillä on adaptiivista potentiaalia eli tarpeeksi geneettistä muuntelua. Risteytyminen on yksi mahdollinen keino kasvattaa populaation geneettistä muuntelua. Tutkielmani tavoite on tutkia lämpötilasopeutumista vertaamalla kahden kekomuurahaislajin, kaljukekekomuurahaisen (*Formica polyctena)* ja tupsukekomuurahaisen (*Formica aquilonia),* sekä niiden risteymien välisiä eroja lämpötilakestävyydessä. Kyseisillä kekomuurahaisilla on poikkeavat levinneisyysalueet: tupsukekomuurahainen on levittäytynyt pohjoiseen Euroopaan kun taas kaljukekomuurahaisen levinneisyyalue on eteläisempi. Keräsin tutkielmani näytteet Etelä-Suomesta ja Ahvenanmaalta, pieneltä alueelta jossa molemmat lajit esiintyvät yhdessä ja risteytyvät. Tutkielmani tavoite oli selvittää, eroavatko vanhemmaislajit lämpötilatoleranssiltaan, mikä heijastaisi myös eroavaisuuksia niiden levinneisyysalueissa. Testasin myös, onko risteymäyksilöiden lämpötilatoleranssi laajempi kuin parentaalilajien, mikä mahdollisesti tarjoaisi edun sopeutumisessa muuttuvaan ympäristöön.

Testasin lämpötilatoleranssia kahden eri kokeen avulla, joissa mitattiin muurahaisten toleranssin ylä- ja alarajoja. Ennustin, että pohjoisempaan levittynyt tupsukekomuurahainen selviää paremmin kylmässä, kun taas eteläisempi kaljukekomuurahainen selviäisi paremmin lämpimässä. Tulokset osoittivat, että parentaalilajit eroavat lämpötilakestävyydeltään ja nämä erot heijastavat lajien välisiä eroja levinneisyysalueissa. Parentaalilajit ovat mahdollisesti sopeutuneet luontaisilla alueillaan erilaisiin lämpöolosuhteisiin, mikä on johtanut eroavaisuuksiin lajien välillä korkeiden ja matalien lämpötilojen sietokyvyssä. Tulokset osoittivat myös, että risteymäyksilöiden lämpötilatoleranssi ei ollut laajempi kuin parentaalilajien. Havaitsin myös, että yksilöiden väliset erot ruumiinkoossa olivat merkitsevä tekijä niiden kuuman sietokyvyssä, isommat yksilöt kestivät kuumuutta paremmin kuin ruumiinkooltaan pienemmät. Tutkielmani tulokset auttavat ymmärtämään risteytymisen mahdollisuuksia ja seurauksia luonnon populaatioille. Kekomuurahaiset ovat avainlaji boreaalisen vyöhykkeen metsissä ja alati haavoittuvaisemmassa asemassa ilmaston muuttuessa ja elinympäristöjen pirtsaloituessa. Tutkielmani tulokset auttavat havainnollistamaan kyseisten lajien populaatioiden suuntaa tulevaisuudessa ilmaston muuttuessa edelleen.

Avainsanat – Nyckelord – Keywords

risteytyminen, Formicidae, hyönteiset, lämpötiladaptaatio, ilmastonmuutos

Ohjaaja tai ohjaajat – Handledare – Supervisor or supervisors

Jonna Kulmuni ja Pierre Nouhaud

Säilytyspaikka – Förvaringsställe – Where deposited

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Muita tietoja – Övriga uppgifter – Additional information

Tiedekunta – Fakultet – Faculty

Faculty of Biological and Environmental Sciences

Tekijä – Författare – Author

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Thermal tolerance of two mound-building wood ants *Formica aquilonia* and *Formica polyctena* and their hybrids

Oppiaine/Opintosuunta – Läroämne/Studieinriktning – Subject/Study track

Organismal and Evolutionary Biology

Työn laji – Arbetets art – Level	**Aika – Datum – Month and year**	**Sivumäärä – Sidoantal – Number of pages**
Master's thesis	May 2020	51 + appendices

Tiivistelmä – Referat – Abstract

Global surface temperature is increasing at an alarming rate. Local populations can cope with the change, if they have adaptive potential to face the new thermal regime. Hybridization with a closely related lineage is one potential source of adaptive genetic variability. My thesis aimed to investigate thermal adaptation by looking into thermal tolerance differences between two mound-building wood ants *Formica polyctena* and *Formica aquilonia* and their hybrids. The two parental species have distinct distributions: *F. aquilonia* can be found in Northern Europe while *F. polyctena* is distributed from Central Europe to Fennoscandia. The samples for this thesis were collected from a relatively small area in southern Finland and Åland Islands. Aim of my thesis was to clarify whether the two parental species have distinct thermal tolerances, which would reflect the differences in their distributions. I also tested whether hybrid individuals have wider thermal limits since they have alleles from both northern and southern parental species and could therefore show adaptive potential.

I tested thermal tolerance differences with two temperature assays: heat-knockdown resistance and chill-coma recovery. I hypothesized that *F. aquilonia* would express more cold-tolerant thermal limits whereas *F. polyctena* would express more heat-tolerant limits. My results showed that the parental species differed in their thermal tolerance and expressed thermal limits which reflected their distribution. These results support the thermal adaptation hypothesis: parental species expressed thermal limits that reflected the thermal environment in their native habitat. The results also showed that hybrids could not combine the thermal tolerance of both parental species as they did not have wider thermal tolerance than parental species. Intriguingly, dry weight had a significant role in thermal tolerance, bigger ants coping better with higher temperatures. These results contribute to building up knowledge on the outcomes of hybridization and the potential that species possess in coping with the environmental change. Wood ants are keystone species in boreal forests and the findings of my thesis shed a light on the changes in population dynamics for these species in the face of global climate change.

Avainsanat – Nyckelord – Keywords

hybridization, Formicidae, insects, thermal adaptation, thermal tolerance, climate change

Ohjaaja tai ohjaajat – Handledare – Supervisor or supervisors

Jonna Kulmuni and Pierre Nouhaud

Säilytyspaikka – Förvaringsställe – Where deposited

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Contribution statement

The following people contributed to this thesis:

Jonna Kulmuni

Raphaël Martin-Roy

Pierre Nouhaud

Elisa Nygård

J.K., P.N. and E.N. contributed to the conception and design of the study. J.K., P.N. and E.N. collected the samples. E.N. planned and carried out the pilot experiments.
J.K., R.M-R., P.N. and E.N. carried out the experiments. E.N. performed laboratory work and statistical analysis.

1 Introduction

1.1 The role of hybridization in nature

Hybridization is defined as reproduction between two genetically differentiated populations producing offspring of mixed ancestry (Barton & Hewitt 1985). The biological species concept relies on reproductive isolation and states that mating between different species cannot produce fertile offspring (Mayr 1963). Evolution of complete reproductive isolation takes time, and different levels of gene flow can often occur between two independently diverging lineages.
Studies with modern genomic tools and whole genome sequencing have revealed past or ongoing hybridization to be a common and widespread phenomenon across taxa in both plant and animal kingdoms (Dowling & Secor 1997; McVay, Hipp & Manos 2017; Rieseberg & Wendel 1993; Slager et al. 2018; Taylor & Larson 2019).

The outcomes of hybridization vary widely, and the role and importance of hybridization in natural populations has been the subject of debate (Seehausen 2004). The potential effects of hybridization vary from hybrid sterility or inviability to increasing genetic variability and adaptive potential in the population (Burke & Arnold 2001). In plants, hybridization has been widely acknowledged to be the source of new genetic material and an important part of the speciation process, but in the animal kingdom its potential positive effects have been frequently questioned in the past (Harrison & Harrison 1993; Mayr 1963; Muhlfeld et al. 2009). Nevertheless, increasing numbers of studies have shown the contribution of hybridization to speciation and adaptation also in animals (Abbot et al. 2013; Heliconius Genome Consortium 2012; Meier et al. 2017). In all its complexity, transferring genetic material across species boundaries is a strong creative evolutionary process that can lead to new combinations of adaptive traits. An outstanding question is whether hybridization could help populations in the face of current environmental change.

Today, numerous species face the imminent effects of human-mediated disturbances to natural habitats. These include habitat destruction, introduction of invasive species and increasing global surface temperature. Ongoing anthropogenic-driven climate change requires local species to migrate or adapt to altering conditions to avoid possible extinction (Scheffers et al. 2016). An essential factor for adapting to new conditions in the long term is an adequate level of genetic variation in the population (Sgrò & Hoffman 2011). Plastic responses (i.e phenotypic and/or behavioral plasticity) or migration to more suitable habitats can help to track changing environments temporarily. But on a longer time scale, only sufficient levels of genetic diversity can allow for an evolutionary response that leads to adaptation (Hamilton & Miller 2016). If not present in the population (standing genetic variation), adaptive genetic variation can arise from de novo mutations and/or hybridization (Barrett & Schluter 2008; Orr 2005). From these processes, hybridization is a significant and relatively fast source of adaptive variation (Hedrick 2013). Hybridization can also result in genomic regions harboring adaptive variation being transferred between diverging lineages. This phenomenon is called ‘adaptive introgression’. Adaptive introgression can fuel adaptation surprisingly fast by combining fitness-enhancing genotypes and therefore possibly save the receiving population from decline,

especially in rapidly changing climate (Kremer et al. 2012; Song et al. 2011; Oziolor et al. 2019). Thus, hybridization could help populations adapt into rapid environmental change.

1.2 Characteristics of thermal adaptation

Increasing global surface temperature is one of the most pressing human-mediated environmental disturbances that populations encounter in their habitat (Lenoir & Svenning 2015; Root et al 2003; Sala et al. 2000). Estimating the effect of global warming on biodiversity requires knowledge on how temperature limits species distributions. Changes in thermal regime have already led to considerable range shifts in many species and taxonomic groups (Devictor et al. 2008; Jump, Mátyás & Peñuelas 2009; Scheffers et al. 2016; Wilson et al. 2005).

Ectothermic insects are among the most diverse taxa on our planet (Wilson 1999). Since these organisms lack internal thermoregulation, their life history traits such as reproduction, growth and longevity, are tightly linked to external temperature. Coping with a novel thermal regime has led to so-called thermal adaptations that can be witnessed across insect taxa (Frazier, Huey & Berrigan 2006; Kingsolver et al. 2011). These adaptations vary from phenological shifts to changes in reproductive behavior and physiological responses (Kong, Hoffman & Kearney 2019; Pritchard, Harder & Mutch 1996). Species' vulnerability to changing climate has been pinned down to certain thermal adaptations that enhance its capability to withstand environmental conditions by thermoregulation. One of these traits is thermal tolerance, which defines the organism's physiological sensitivity to temperature change (Angilletta 2009). However, thermoregulation always comes with a high energetic cost (Huey & Slatkin 1976). This has led to the thermal adaptation hypothesis, which states that organisms should express thermal tolerance limits adapted to their local surroundings (Sunday et al. 2011). Therefore, organisms living in warmer environments must display higher limits of warm tolerance than organisms living in colder environments and vice versa.

Hybridization has been shown to fuel thermal adaptation across taxa, for example, by creating ecological novelties or shaping physiological responses (Martin 2019; Pereira, Barreto & Burton 2014). If hybridizing taxa differ in their thermal preferences, hybridization can also

lead to environmentally-dependent representation of both parental genomes in hybrids (Smukowski et al. 2019), which could also be an adaptive response to changes in the thermal regime (Martin-Roy & Kulmuni 2019).

1.3 Wood ant ecology and conservation

Many species of Hymenoptera (ants, bees and wasps) are social insects that have hierarchical societies in their colonies. Ants (Hymenoptera: Formicidae) are an abundant taxonomic group that can be found in almost every continent (Fisher 2010). In the majority of ant colonies, reproductive effort is the queen's responsibility while workers tend the offspring and take care of the nest. Females (queens and workers) arise by sexual reproduction and develop from fertilized diploid eggs, whereas males develop from unfertilized eggs and are therefore haploid. The haplodiplontic life cycle and sociality of ants provides unique potential for studying evolutionary processes, such as hybridization (Nouhaud et al. 2020) . This allows studying, for example, recessive alleles that are expressed in haploid males but which are masked in diploid females when heterozygous.

In southern Finland, the two mound-building wood ant species *Formica polyctena* and *Formica aquilonia* naturally hybridize and these hybrid populations are maintained over the years (Kulmuni et al. 2010). The two parental species *F. aquilonia* and *F. polyctena* have likely diverged in allopatry approximately 500 000 years ago and have distinct distributions: *F.aquilonia* can be found in Northern Eurasia and in Central Europe while *F.polyctena* is distributed from Central Europe to Fennoscandia and Russia (Goropashnaya et al. 2004, Fig.1).

F. polyctena and *F. aquilonia* belong to the *Formica rufa* group of wood ants, which can be classified as keystone species and ecological engineers in forest ecosystems (Sorvari 2016). These species contribute to nutrient cycling, food web structure, seed dispersion, pest control and the energy cycle in the forest (Stockan & Robinson 2016). Wood ant species require suitable microclimatic conditions and large habitat patches for their colony construction, which makes them vulnerable to clearcuts and forest fragmentation (Sorvari 2016). Colonies that existed in the forest are rare to colonize the same area again after a clearcut (Sorvari &

Hakkarainen 2007). Regardless of exposure to multiple threats, conservation status of wood ants is often underestimated because of large census population sizes. However, the effective population size is relatively small compared to solitary insects because only queens and males are responsible for reproduction (Gyllenstrand & Seppä 2003; Romiguier et al. 2014).

As social insects, ants are affected by external temperature both at the individual and whole colony levels. Temperature regulates to a great extent their life cycle, from brood development to timing of reproduction and colony maintenance. Nevertheless, ants are not entirely at the mercy of environmental temperature. Because of their sociality, a large number of individuals can regulate temperature in their immediate surroundings in the nest (Jones & Oldroyd 2006). The colony can regulate warming and cooling with metabolic heat of individuals and nest construction (Kadochová & Frouz 2013). Adaptation to different environmental conditions has led to a considerable diversity in the thermal tolerance of ants (Kaspari et al. 2015). Variability in thermal tolerance shapes the distribution of ant species on latitudinal and elevational gradients. Warm tolerance decreases with latitude, and species in high elevations show increased cold tolerance (Bishop et al. 2017; Diamond et al. 2012).

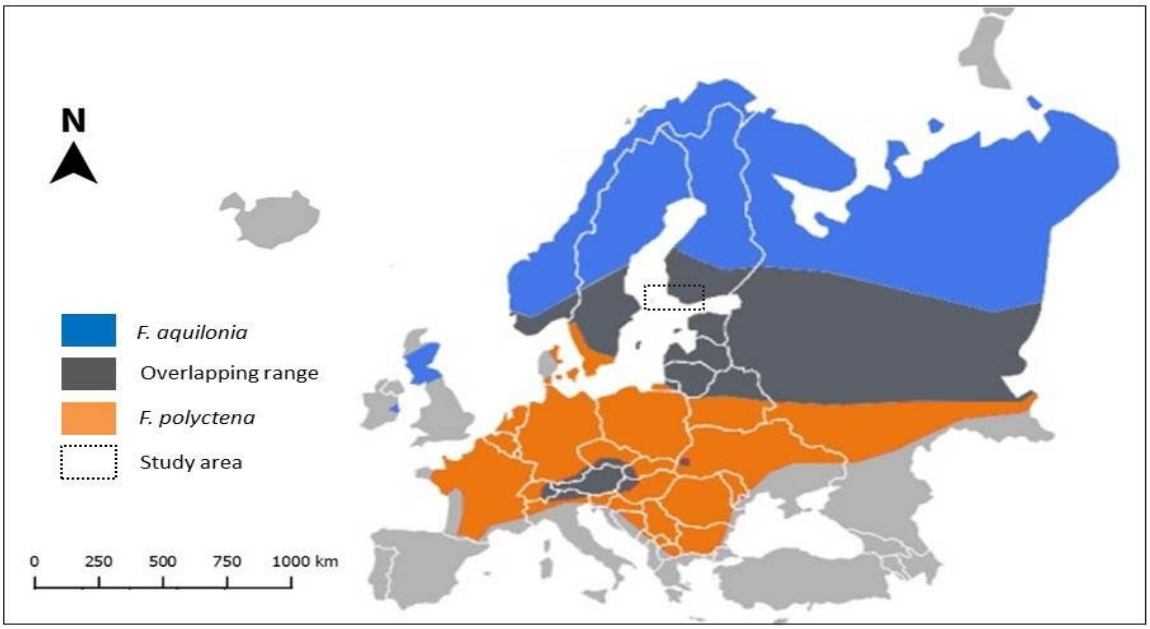

Figure 1. Map of the distributions of *F. aquilonia* and *F. polyctena* and their overlapping range (adapted from Stockan & Robinson 2016).

1.4 Aims and research questions

In my MSc project I investigate thermal tolerance of finnish *F. aquilonia*, *F. polyctena* and their hybrids. Northernmost-distributed parental species *F. aquilonia* should be more cold-tolerant, whereas southernmost-distributed *F. polyctena* could be adapted to warmer environments and would be more heat-tolerant. The aims of my project are to 1) study whether the two parental species differ in their thermal tolerance, which could be expected due to their current distinct distributions (Fig. 1) and 2) test whether the finnish *F. aquilonia* x *F.polyctena* hybrid individuals have a different thermal tolerance compared to the parental species. I am also testing if 3) the finnish *F. aquilonia* x *F.polyctena* hybrids have wider thermal tolerance compared to finnish populations of the parental species, which would allow them to adapt to changing temperatures.

Research questions

1) Do parental species differ in their thermal tolerances?
2) Do hybrids have a different thermal tolerance compared to the parental species?
3) Do hybrids have a wider thermal tolerance compared to the parental species, potentially allowing adaptation to changing temperature regimes?

To answer these questions, I will combine temperature assays and population genetic analyses with parental and hybrid ants from different populations. I am predicting that parental species differ in their thermal tolerance due to their current distributions, based on the thermal adaptation hypothesis (Sunday et al. 2011). I am also expecting that the hybrid individuals will have wider temperature tolerance since they carry alleles from both parental species. Therefore, hybrid individuals could adapt to a wider thermal regime than parental species. The results from my thesis will contribute to reveal how these species and their naturally occurring hybrids cope with changing environmental conditions. They will also help to predict if hybrids could have better adaptive potential compared to parental species in the face of climate

change. Clarifying these species' response to changing temperatures is a major issue, especially regarding the conservation of keystone species like Finnish wood ants.

2 Materials & Methods

2.1 Sampling of colonies

In order to measure their thermal tolerance, we collected samples from finnish populations of the two parental species *Formica aquilonia* (Aq) and *Formica polyctena* (Pol) along with *F. aquilonia* x *F. polyctena* hybrids (Hyb) between 2nd and 6th of May 2019. We chose adult workers for the experiment, since they can be collected in large numbers in each nest to reach sufficient statistical power. To avoid any potential population effect, we sampled three populations for each group (two parental species and hybrids), and three nests per population (except for the Långholmen hybrid population, where we sampled five nests). Two parental *F. polyctena* populations were collected from the Åland Islands, whereas all remaining populations were situated in Southern Finland (Fig. 2). For each nest, we sampled individual workers with nest material in the 20 cm topmost layer of the nest. We collected approximately 200 individuals from each nest and kept samples in 2l Minigrip bags with nest material during transportation.

Overall, we used 10 populations and 29 nests in the experiments (9 *F. aquilonia*, 9 *F. polyctena* and 11 hybrid nests). Identification of the nests was based on previous genetic studies using microsatellite markers (Kulmuni et al. 2010, Beresford et al. 2017) and was confirmed later with my own genotypic analysis (see below).

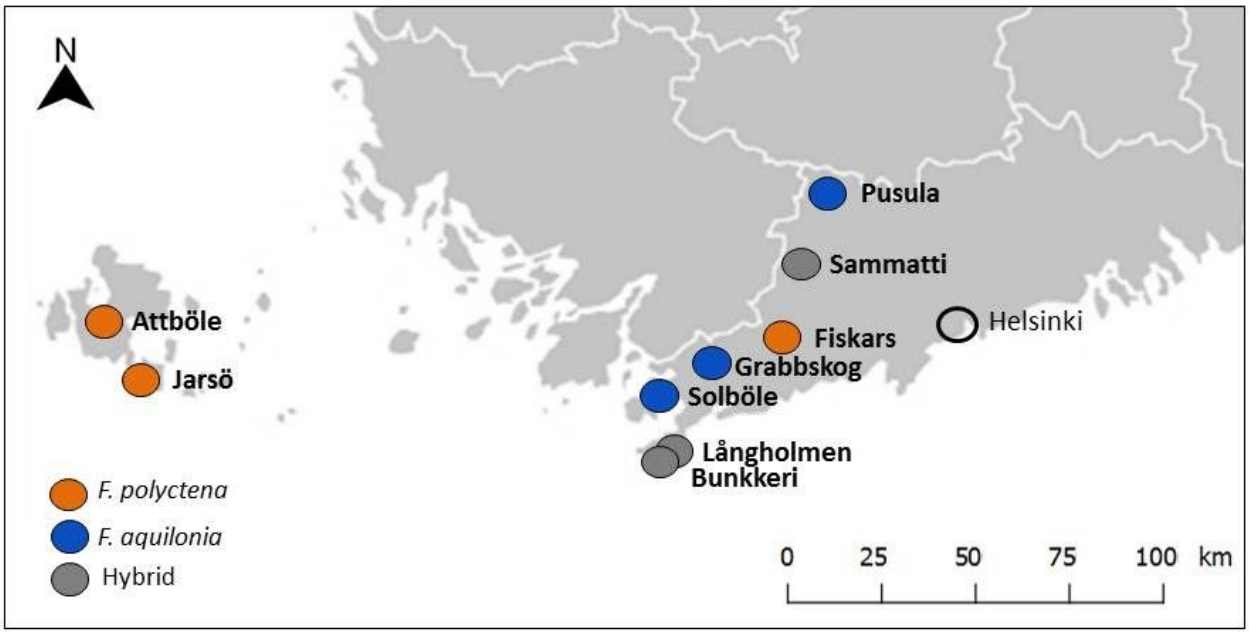

Figure 2. Map of sampling locations in southern Finland and the Åland Islands. The location of all sampled populations (nine altogether) from three different groups are indicated in grey (hybrids), orange (*F. polyctena*) and blue (*F. aquilonia*).

2.2 Colony maintenance

After collection, we brought the samples to Tvärminne Zoological Station, where artificial nests were set up using open plastic nest boxes (26 x 40 x 16cm). We discarded excess workers to prevent overcrowding. Walls of the nest boxes were coated with Fluon to prevent ants from escaping. All nests had a water-filled Eppendorf tube with a cotton plug for hydration and moss (*Sphagnum* spp.) to maintain moisture. We kept the nest boxes under stable conditions at +20°C and a light cycle of 14h light/10h dark. We fed the colonies daily ~5ml standard ant diet of agar, eggs and honey (Bhatkar & Whitcomb 1970). To keep moisture stable in nest boxes, we sprayed the nests daily with water. All nests were under the same conditions for twelve days until the onset of the experiments.

2.3 Experimental design

Temperature assays

We examined temperature tolerance with two indicators: heat-knockdown resistance and chill-coma recovery. These are two widely used metrics in the study of insect thermal adaptation (Jørgensen, Malte & Overgaard 2019, Teets et al. 2019, Angilletta 2009). Knockdown resistance is defined as the time required for ants to lose mobility at high temperatures whereas chill-coma recovery is the recovery time from prolonged exposure to extremely low temperatures (Huey et al. 1992). We estimated the critical thermal maximum (CTmax) in a preliminary experiment to determine a challenging temperature for the knockdown resistance assay (see below). CTmax can be described as a stressful temperature where individuals rapidly lose coordinated muscle control and no motility response is detected (Huey et al. 1992).

I collected all individuals after thermal experiments and stored them in 1.5ml Eppendorf tubes with ethanol for later genotypic analysis.

Critical thermal maximum

Critical thermal maximum (CTmax) was determined in a preliminary experiment. We fed and watered the ants the previous evening before the experiments. We randomly chose six ants from each group (Aq, Pol, Hyb) from twelve randomly picked nests (72 individuals overall). We sorted the ants individually on petri dishes (⌀60mm) to minimize the effect of interactions between individuals. All petri dishes contained cotton wool saturated with water to prevent desiccation. Immediately after collection, we moved the Petri dishes to a walk-in climate chamber with stable temperature.

During the experiments, we tapped the dishes periodically every five minutes, to check if any motility response could be detected.WeI observed the ants slowly falling into a coma and

not moving but showing some signs of response (e.g., antenna movement) for long before complete loss of motor functions. The time of immobilization was marked when ants did not respond to light tapping anymore (no movement). The CTmax experiment was repeated with three different room temperatures: 41 °C, 46 °C and 51 °C. During each trial, we monitored room temperature with temperature loggers and checked surface temperature every 20 minutes. After three trials, we determined 51 °C to be CTmax, since all samples immobilized within the first 27 minutes of the experiment.

Heat-knockdown resistance

Ants were fed and watered the previous evening before the experiment. Based on results from the CTmax experiment, room temperature of 46 °C was used in the heat-knockdown resistance experiment. This temperature was a few degrees lower than CTmax and challenging enough for the ants to immobilize within a reasonable amount of time. Random workers sampled from the top of each nest box were placed each on their own petri dish (⌀38mm, see Fig. 3). We used ten workers from each nest in the experiment, altogether 300 individuals. Petri dishes had water-saturated cotton wool for hydration. Dishes were organized on eight trays randomly, to minimize the effect of any potential temperature gradient in the climate chamber. Sampling of the ants to the petri dishes was done within a period of one hour. Knockdown resistance was measured in a single session, with all ants being observed simultaneously.

Four observers scanned the dishes during the experiment and switched places between trays every 30 minutes to minimize observer bias. Tapping of the dishes was done every 10 minutes in a systematic manner. Observers marked down the time when no signs of response to light tapping was detected (Fig. 3). Since falling into a coma was a long process, individuals that were not moving but waved antennas or legs were also marked. Ants were observed drinking from the water-saturated cotton wool during the experiment. Observers checked room and surface temperatures every 10 minutes. Mean room temperature during the experiment was 45°C. The experiment lasted four hours, after which we collected all individuals and stored them in 1.5ml Eppendorf tubes with ethanol for later genotypic analysis.

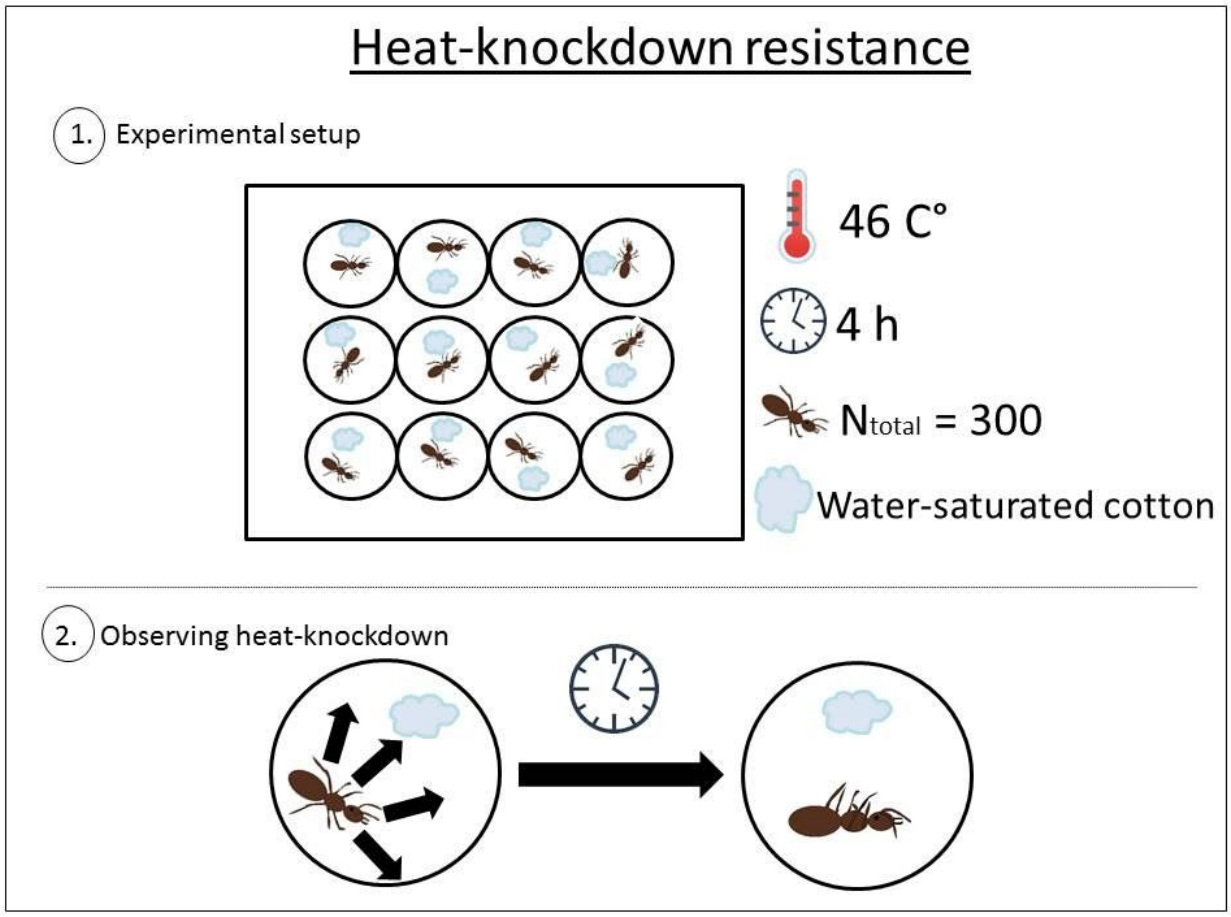

Figure 3. Experimental design in the heat-knockdown experiment. In the setup (1), workers were individually placed in petri dishes that were kept in a climatic chamber at 46℃ for 4h. The heat-knockdown time was defined (2) when individuals were immobilized and did not respond to light tapping of petri dishes.

Chill-coma recovery

To detect a challenging temperature for the chill-coma recovery assay, we tested different temperatures and cold treatment durations in a preliminary experiment. Finally, 25 minutes in -13°C degrees was chosen to be assayed in the chill-coma recovery experiment. Ants were fed

and watered in the evening before the experiment. Following the sample scheme of the heat-knockdown resistance assay, we assayed ten workers from each nest, altogether 300 individuals. Chill-coma recovery was measured in two batches, both with 150 ants. Both trials were carried out on the same day, the first one starting at 16.00 and second at 20.00. Five workers per nest were sampled and placed each on their own petri dish (⌀38mm) from the top of each nest. Dishes were sorted randomly on five trays, to minimize the effect of temperature gradient in the climate chamber. Sampling of the ants was done in 30 minutes. Trays were moved to a walk-in climate chamber with a stable temperature of -13 ± 2 °C. Trays were kept in the chamber for 25 minutes, after 22 minutes each dish was gently tilted so that all ants were on the left side of the dish when trays were taken out of the chamber (Fig. 4). Trays were moved to room temperature (23 °C) and placed on a table. Chill-coma recovery was measured from the time when trays were taken out of the climate chamber until the ant woke up and walked over a line drawn across the middle of each dish (Fig. 4). This was considered indicative of recovery as ants are generally known to immediately start exploring their surroundings when recovered (Angilletta et al. 2007). For both batches the experiment was recorded on video (so that simultaneous recovery events could be recorded precisely) and recovery times were documented by a single observer afterwards. All trays were recorded for 30 minutes and ants that did not move over the line during that time period were considered dead.

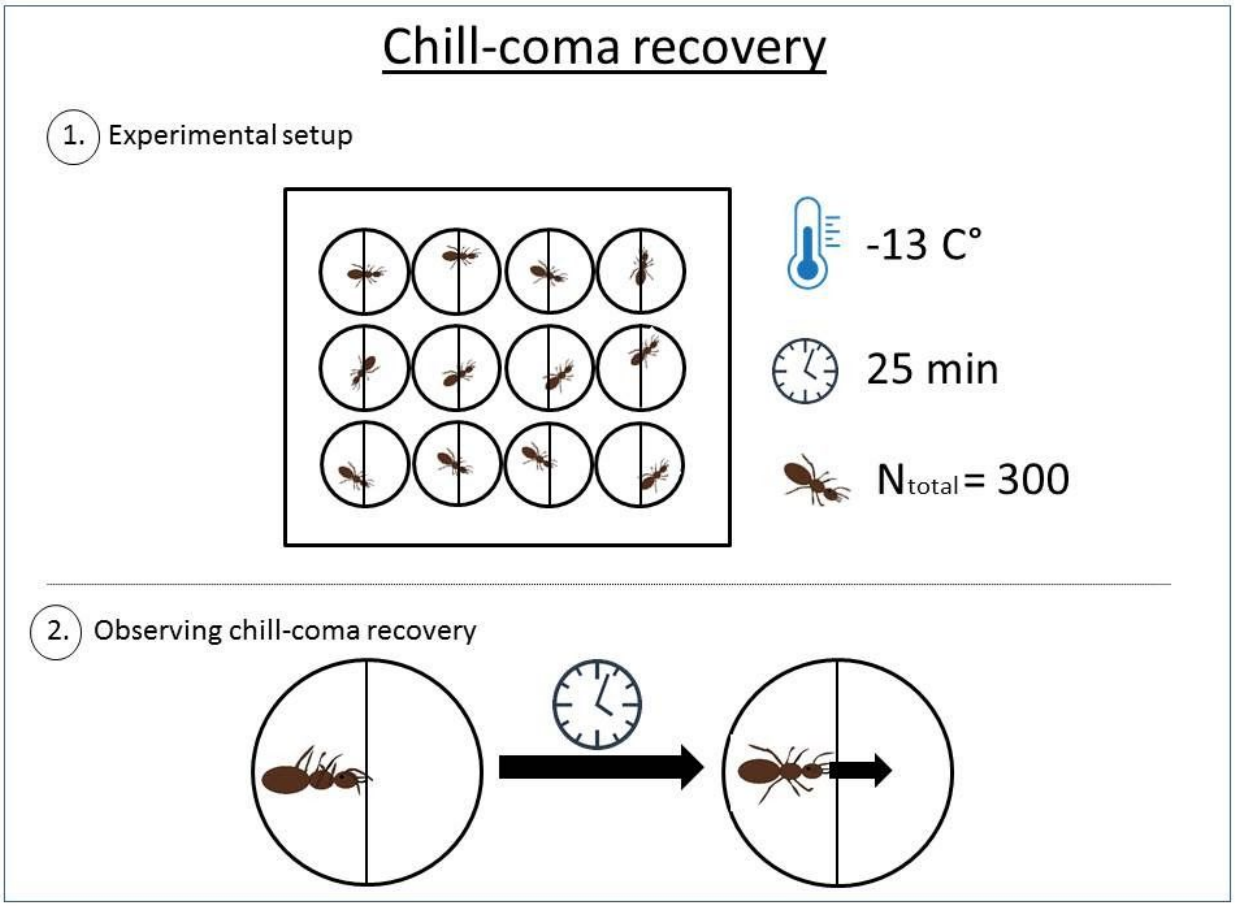

Figure 4. Experimental design in the chill-coma recovery experiment. The same experimental setup was repeated in two batches, both with 150 individuals. In the setup (1), workers were individually placed on petri dishes and kept at - 13℃ for 25min. After this, the dishes were brought to room temperature and the recovery was recorded on video. The chill-coma recovery was observed (2) when the ant woke up and walked across a line drawn across the center of the Petri dish.

2.4 Laboratory analysis

Dry weight

Because size may influence thermal tolerance, six individuals per nest were weighed from each group (54 *F. aquilonia* individuals, 54 *F. polyctena* individuals and 66 hybrid individuals). I dried

the samples briefly on a tissue to remove the excess ethanol. Individuals were moved to new Eppendorf tubes and let in a drying oven (50 ℃) for five hours, after which dry weight was measured individually on a laboratory scale with precision of 0,0001g. We weighed only samples from the heat-knockdown experiment.

DNA extraction and genotyping

To verify the group identity (Aq, Pol or Hyb) of each nest, I used microsatellite markers to genotype six individuals from each nest. DNA extraction was done from the samples used for dry weight measurements (i.e., only samples from the heat-knockdown experiment).

I performed purification of total DNA with DNeasy Tissue kits (Qiagen) following the manufacturer's spin-column protocol for insects. Samples were genotyped based on previous genetic studies using nine microsatellite markers and PCR conditions determined in earlier studies; Fe7, Fe17, Fy3, Fe19, Fe13, Fy15, Fy12, Fl29, and Fy13 (Kulmuni et al. 2010, Beresford et al. 2017). Total DNA was amplified with polymerase chain reaction (PCR) in Veriti 96-well Thermal Cycler (Applied Biosystems) with fluorescent labelling. Genotypes were resolved by capillary electrophoresis with a 3730 DNA Analyzer (Applied Biosystems) using 500 ROX size standard. Lastly, genotypes were scored with GENEMAPPER version 4.0 (Applied Biosystems). As genotypes were later compared to samples genotyped in earlier study (Beresford et al. 2017) I used three reference samples of known genotypes in every PCR reaction to control for variation potentially introduced by different PCR and DNA Analyzer machines.

2.5 Statistical analysis

Species identity was confirmed using principal coordinates analysis (PCoA) of the genotypic data with GENALEX (Peakall & Smouse 2006). To study the genetic structure of my samples, I first used PCoA which is based on pairwise genetic distance between samples. I calculated pairwise genetic distance between nests pooling individual worker genotypes within a nest

together. I compared data from a previous study (Beresford et al. 2017) to my own samples, to check my samples while scoring the genotypes with GENEMAPPER.

To identify population structure and to further verify the classification of samples into Aq, Pol and Hyb groups, genotypes were analyzed with STRUCTURE 2.3.4 (Pritchard, Stephens & Donnelly 2000) varying the number of genetic clusters (K) from 1 to 10. I used an admixture model with independent allele frequencies and ran five iterations for each K-value with burn-in of 2,000,00 and 1,000,000 MCMC replicates after burn-in. ΔK statistics (Evanno, Regnaut & Goudet 2005) were calculated with STRUCTURE HARVESTER (Earl 2012) to detect the uppermost hierarchical level of structure in the data.

Differences in chill-coma recovery and knockdown resistance times between the groups were compared with Kaplan-Meier survival curves and Cox proportional hazards model from *survival* and *survminer* packages in R (R Core Team 2013, software v. 3.6, Therneau & Grambsch 2000). I excluded from the analysis two individuals who lacked water-saturated cotton during the heat-knockdown experiment. To control for correlated results between individuals from the same nests, I used a robust sandwich estimate of variance in the survival analysis. I used the group status and dry weight as covariates in the Cox proportional hazards model. Because dry weight was measured from samples from the heat-knockdown resistance experiment, mean weight per nest was calculated to include dry weight in the analysis of the chill-coma recovery experiment.

Since a large number of individuals were alive at the end of the heat-knockdown experiment and a significant amount did not recover from the cold treatment, I tested survival differences also with binomial logistic regression models, building a generalized-linear mixed model with *glmer* from *lme4* package in R (Bates et al. 2014). To explore factors that were affecting survival, I created a binary variable with "1" representing survival (heat-knockdown) or recovery (chill-coma recovery) and "0" death during experiment and used this as outcome variable in the logistic regression. Because of the hierarchical nature of my data, I added the population of origin as a random effect to the model. Independent variables were group status and mean dry weight per nest.

3 Results

3.1 Genetic structure and identity of the nests

I studied the genetic structure of my samples to ensure that they were assigned to the right group status in the analysis. Nests were assigned to finnish *F. aquilonia*, *F. polyctena* and hybrids based on the combination of a principal coordinates analysis (PCoA), the analysis of population structure (STRUCTURE) and prior knowledge from the same populations, if available (Beresford et al. 2017). Towards this end, PCoA with pairwise genetic distance between the nests included microsatellite data from a previous study (Beresford et al. 2017) to verify that my assignments corresponded to previously identified group (Aq, Pol, Hyb) status. Finnish *F. polyctena* populations from Åland Islands had not been collected before, and therefore lacked comparison.

The first axis of PCoA explained 30.8% of the variation and divided data into two parental groups with hybrids being intermediate (Fig. 5). The right end of axis 1 contained only nests from the parental *F. aquilonia*, with the exception of one nest from the Sammatti population which was previously assigned as a hybrid nest (Beresford et al. 2017). The left end of axis 1 contained parental *F. polyctena*, and also two nests from the Sammatti population. The distinction between *F. polyctena* and hybrid nests was not as clear as the distinction between between *F. aquilonia* and hybrid nests.

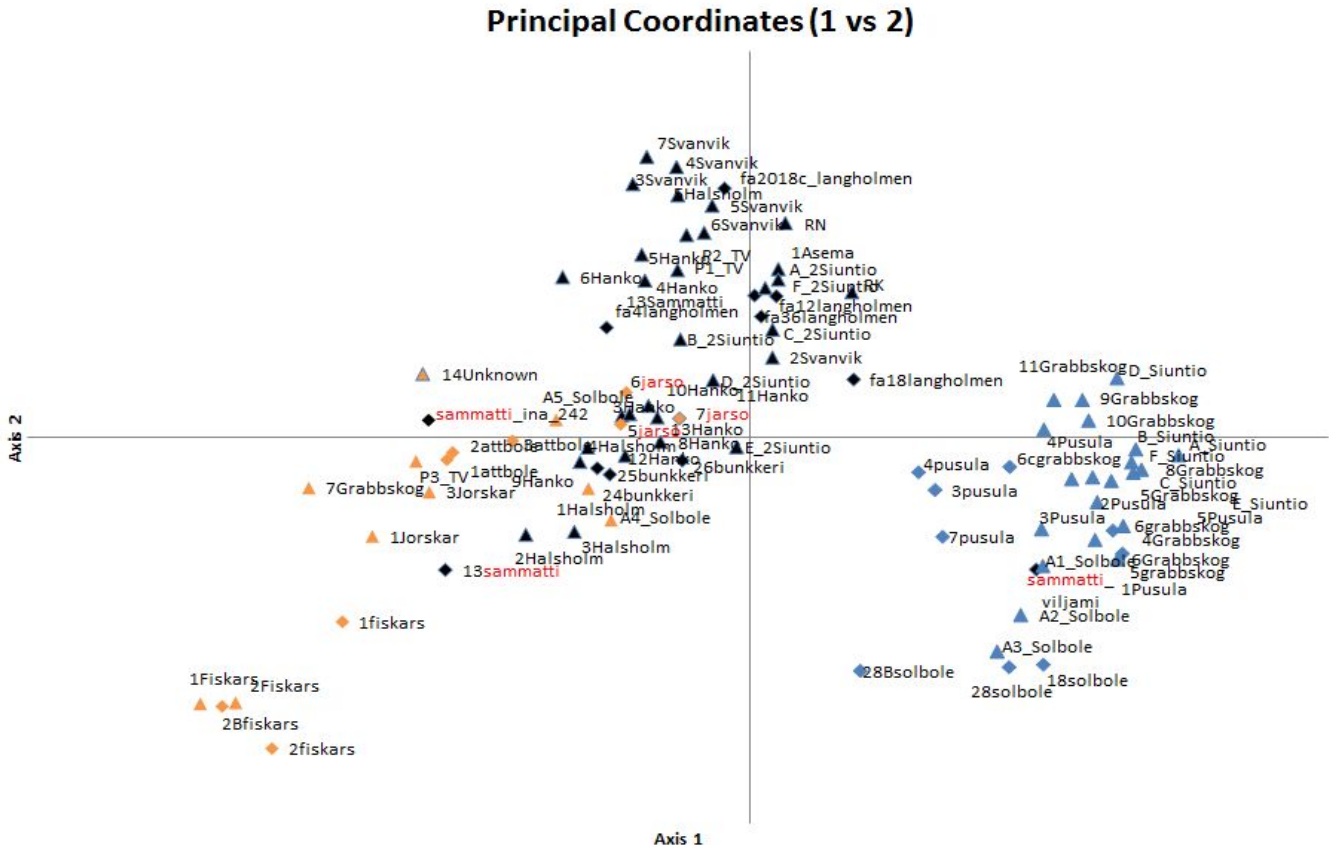

Figure 5. Principal coordinates analysis of nine microsatellite markers from 29 new nests (diamonds) and 66 previously sampled nests (triangles, Beresford et al. 2017). The data points are colored based on the initial identification of the nests (orange for *F. polyctena*, blue for *F. aquilonia* and black for hybrids). The first axis explains 30.8% of the variation and the second axis 10.3%. The first axis divides the samples according to parental gene pools, where finnish *F. polyctena* individuals are situated to the left, finnish *F. aquilonia* to the right and hybrids in the middle. Nests that were assigned to different groups than in Beresford et al. (2017, nests from Sammatti) are shown in red. Three nests from Jarsö that were initially classified as *F. polyctena* are also shown in red.

Population structure was checked with STRUCTURE, using K-values ranging from 1 to 10. The ΔK-statistic analysis implemented in STRUCTURE HARVESTER diagnosed K=2 as the most likely number of genetic clusters that best fits the data (Fig. 6). The two clusters in STRUCTURE

analysis present finnish populations of *F. aquilonia* and *F. polyctena*. There is different levels of admixture in the hybrid nests, as shown in the STRUCTURE plot in figure 7. Similarly as in PCoA, STRUCTURE results suggest the one nest from Sammatti (Sammatti_Viljami) mostly shows finnish *F.aquilonia* ancestry. Two nests from Sammatti that were situated in the finnish *F. polyctena* parental gene pool in PCoA also cluster with finnish *F. polyctena* individuals in the STRUCTURE plot. The population from Jarsö seemed to be more admixed than other populations from the finnish *F. polyctena* parental gene pool.

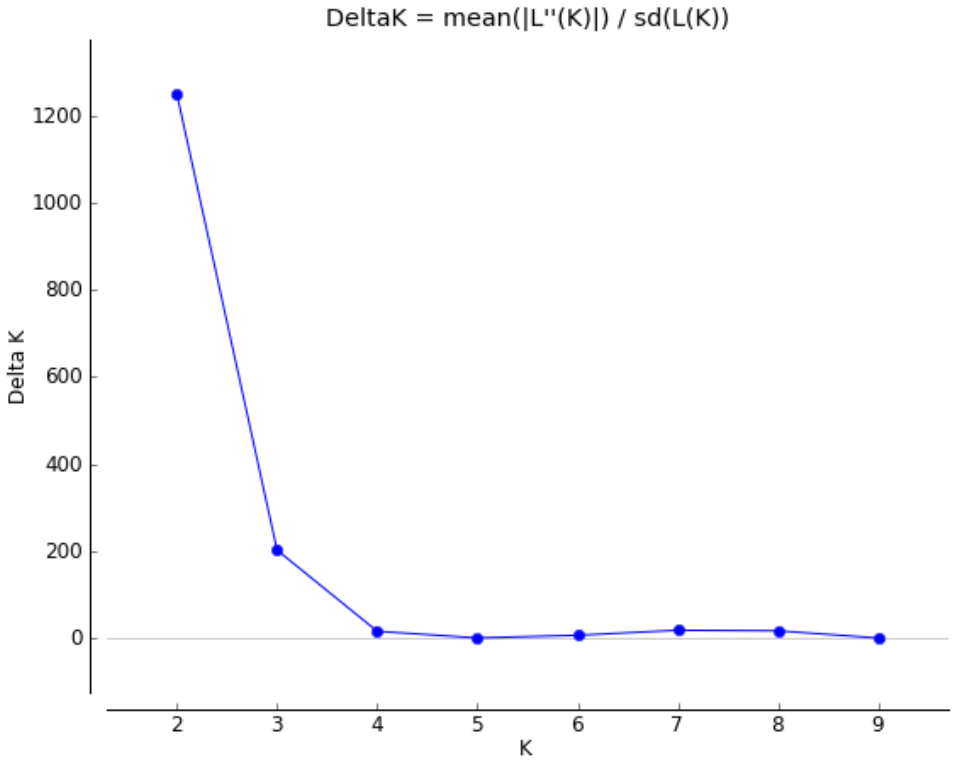

Figure 6. Results of the ΔK-statistics from STRUCTURE HARVESTER. STRUCTURE was run with the number of genetic clusters (K) ranging from 1 to 10 and five iterations per run. K=2 has the highest ΔK value, suggesting this is the uppermost hierarchical level of structure in the individual-level microsatellite data.

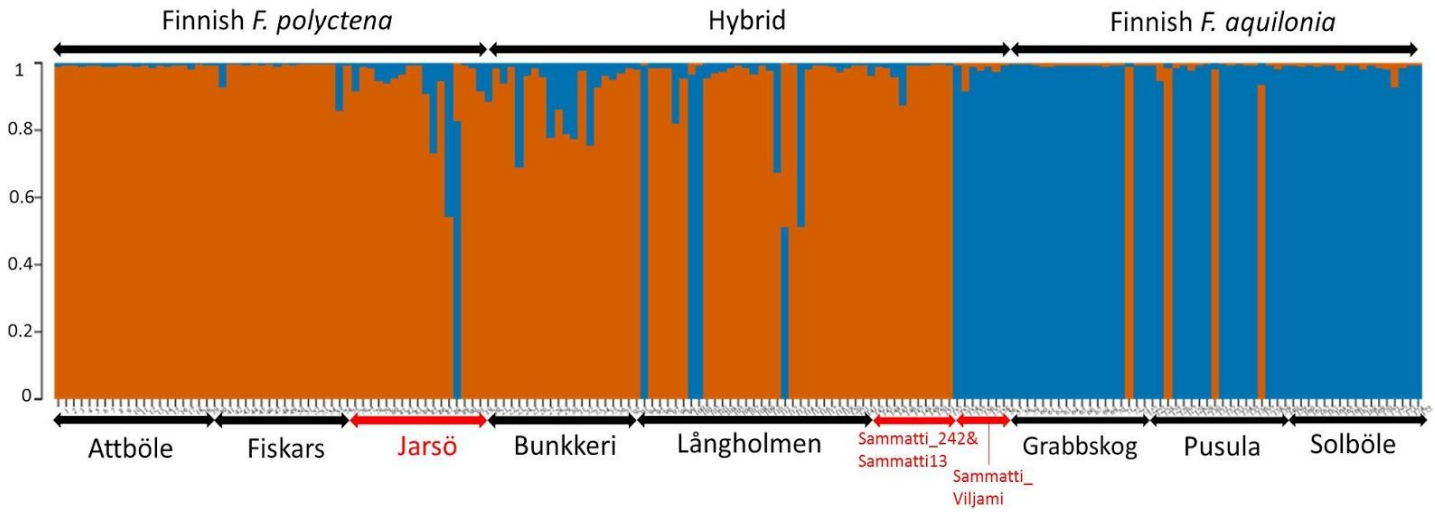

Figure 7. Results from the STRUCTURE assignment of 175 individuals. The three groups are identified into clusters with different levels of finnish *F. aquilonia* (blue) or finnish *F. polyctena* (orange) origin. Finnish *F. polyctena* populations are on the left, hybrids in the middle and finnish *F. aquilonia* on the right. Different populations are visualized in the y-axis with black arrows. Y-axis indicates the percentage of assignment of each individual to the two clusters. Two nests from Sammatti (Sammatti_242 & Sammatti13) show signatures of finnish *F. polyctena* origin, whereas Sammatti_Viljami clusters with finnish *F. aquilonia* individuals. The population from Jarsö shows a signature of mixed ancestry.

Except from the Sammatti and Jarsö populations, there were no significant changes in the genetic makeup of the sampled nests in comparison to the previous results from the same population (Beresford et al. 2017) based on the PCoA and STRUCTURE. Therefore, for all samples collected for this study except the populations from Sammatti and Jarsö I kept the initial assignments from Beresford et al. (2017, Table 1). Sammatti nests were assigned as one finnish *F. aquilonia* nest and two finnish *F. polyctena* nests. I defined the population from Jarsö as a hybrid based on the results of the PCoA and the STRUCTURE analysis.

Table 1. Assignment of genotyped nests to finnish *F. polyctena* (Pol), *F. aquilonia* (Aq) or hybrid (Hyb). Identification was based on PCoA (Fig. 5) and STRUCTURE analysis (Fig. 7). Nests from previous study were identified by either morphology or genetics (Beresford et al. 2017).

Population	Nest ID	Previous identity	Assigned to
Attböle	ATTBÖLE1	Pol	Pol
Attböle	ATTBÖLE2	Pol	Pol
Attböle	ATTBÖLE3	Pol	Pol
Fiskars	FISKARI1	Pol	Pol
Fiskars	FISKARI2	Pol	Pol
Fiskars	FISKARI2B	Pol	Pol
Jarsö	JARSÖ5	Pol	Hyb
Jarsö	JARSÖ6	Pol	Hyb
Jarsö	JARSÖ7	Pol	Hyb
Grabbskog	GRABBSKOG5	Aq	Aq
Grabbskog	GRABBSKOG6	Aq	Aq
Grabbskog	GRABBSKOG6C	Aq	Aq
Pusula	PUSULA3	Aq	Aq
Pusula	PUSULA4	Aq	Aq
Pusula	PUSULA7	Aq	Aq
Solböle	SOLBÖLE28_2018	Aq	Aq
Solböle	SOLBÖLE28	Aq	Aq
Solböle	SOLBÖLE28B	Aq	Aq
Långholmen	FA04	Hyb	Hyb
Långholmen	FA12	Hyb	Hyb
Långholmen	FA18	Hyb	Hyb
Långholmen	FA36	Hyb	Hyb
Långholmen	FA2018c	Hyb	Hyb
Bunkkeri	BUNKKERI24	Hyb	Hyb
Bunkkeri	BUNKKERI25	Hyb	Hyb
Bunkkeri	BUNKKERI26	Hyb	Hyb
Sammatti	Sammatti_Ina_242	Hyb	Pol
Sammatti	Sammatti_13	Hyb	Pol
Sammatti	Sammatti_Viljami	Hyb	Aq

3.2 Dry weight differences

Individual dry weights ranged from 1.1 milligrams to 4.7 milligrams (Fig. 8). A one-way ANOVA test was conducted to compare the differences in dry weights between groups. There was a

statistically significant difference between groups as determined by a one-way ANOVA ($F(2,295) = 51.01$, $p < 0.001$). According to Tukey's honestly significant difference (HSD) post hoc test, there was a significant difference ($p < 0.001$) between the mean dry weight of the *F. aquilonia* (M=2.14 mg, SD=0.323) and *F. polyctena* (M=2.75 mg, SD=0.445). A post hoc Tukey test showed also that the hybrids (M = 2.66 mg, SD = 0.549) differed significantly ($p < 0.001$) from *F. aquilonia*. Differences in dry weights between *F. polyctena* and the hybrids were not significant (p-value = 0.18).

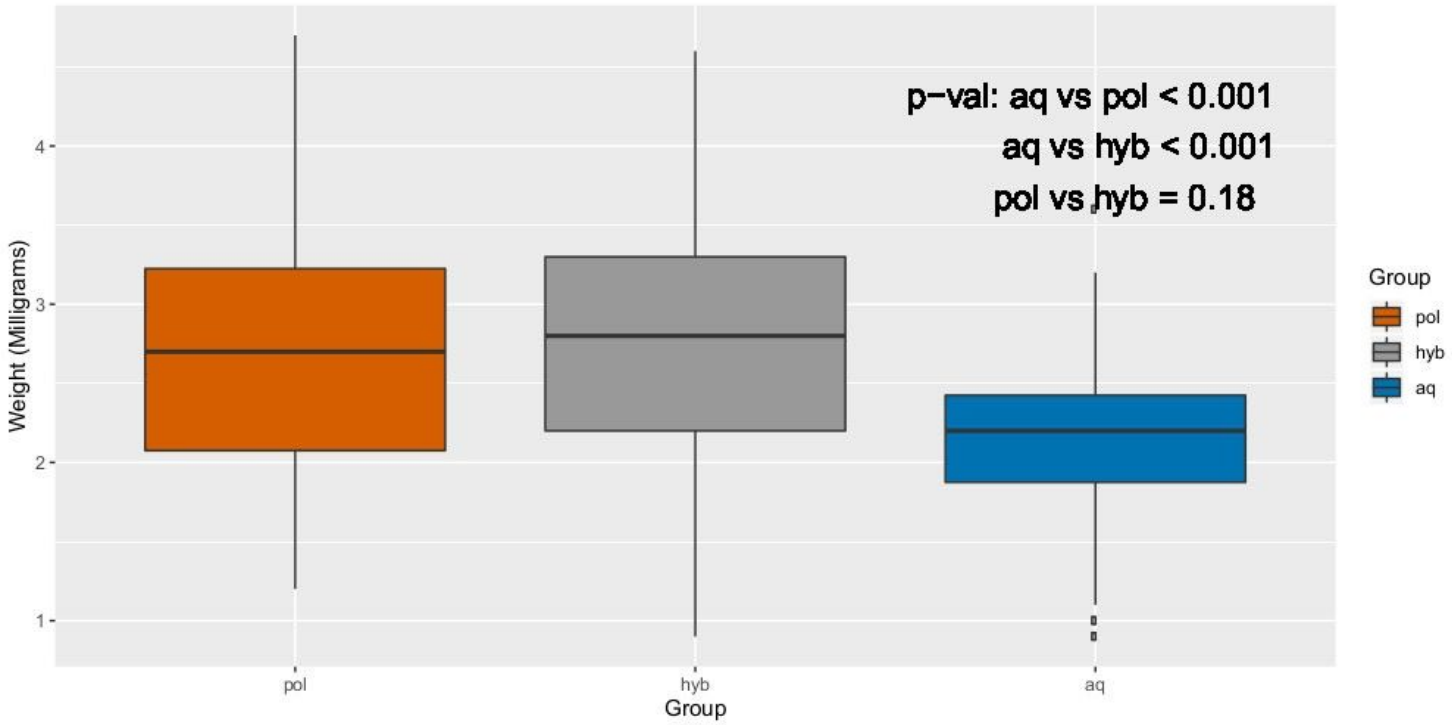

Figure 8. Distribution of individual weights among three groups: finnihs *F. aquilonia* shown in blue, *F. polyctena* in orange and hybrids in grey. Six individuals per nest were weighed from each group, 60 workers from *F. aquilonia*, 48 *F. polyctena* and 66 hybrids.

3.3 Heat-knockdown resistance

To study the prediction that finnish *F. polyctena* is more heat-tolerant than *F. aquilonia*, ten ants per nest (Ntotal=300) were exposed to 51 °C and their activity was recorded for four hours. Finnish *F. polyctena* individuals survived 17% longer in the heat than *F. aquilonia* (M±SD = 171±6.8 and 155±5.9 min for *F. polyctena* and *F. aquilonia*, respectively). According to multiple log-rank tests between the groups, survival rates differed significantly between the ants from *F. aquilonia* and *F. polyctena* (p-value < 0.001, log-rank test, Fig.9). Hybrid ants survived longer than *F. aquilonia* (M±SD = 191±5.3 min for hybrids) but had no difference in survival probability compared to *F. polyctena* (p-value = 0.05, log-rank test, Fig.9). There was a considerable variability in survival between populations, but the differences between groups were not driven by a subset of populations (Fig. S1).

A Cox proportional hazards model was constructed to study the effect of different covariates on survival in the heat-knockdown experiment. Mean dry weight per nest had a highly significant positive effect on the survival probability (p-value < 0.001, chi-square test, Table 2). Therefore, bigger workers survived longer under heat conditions (Fig. 10). Interaction term between mean weight and the group status was not a significant addition to the model (p-value=0.75, ANOVA, Table 2). These results were also consistent in the mixed effects logistic binomial model (Table 3).

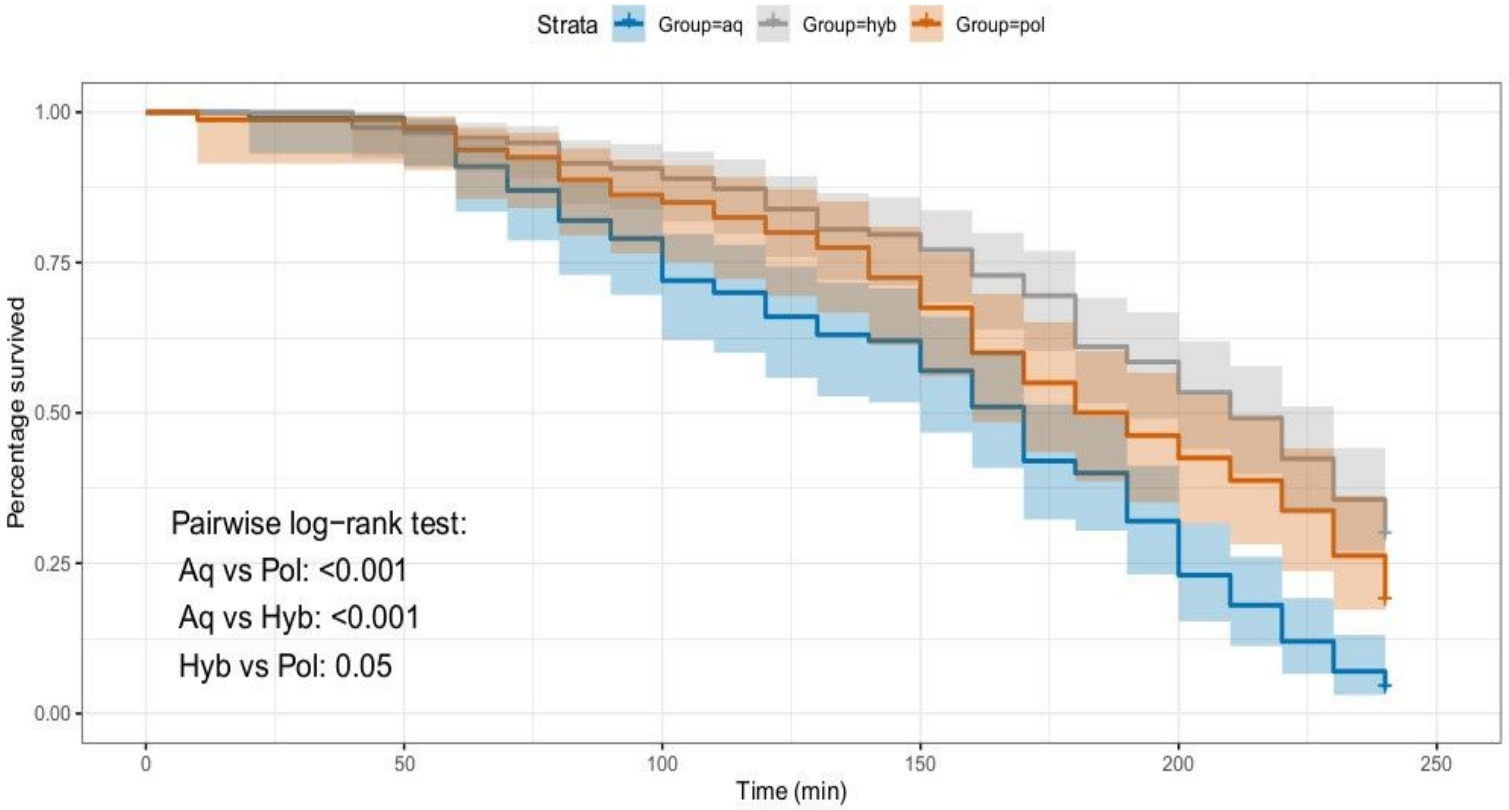

Figure 9. Kaplan-Meier survival curves from the heat-knockdown resistance experiment. The x-axis indicates the time from the beginning of the experiment and the y-axis the percentage of individuals alive at certain time points. *F. aquilonia* is indicated with a blue curve, *F. polyctena* in orange and hybrids in grey. The 95% confidence interval of the curve is highlighted with a lighter color. The *F. aquilonia* group has the steepest curve and individuals start to fall into heat-coma quickest. Hybrids and *F. polyctena* survive longer and there is no significant difference between them in survival.

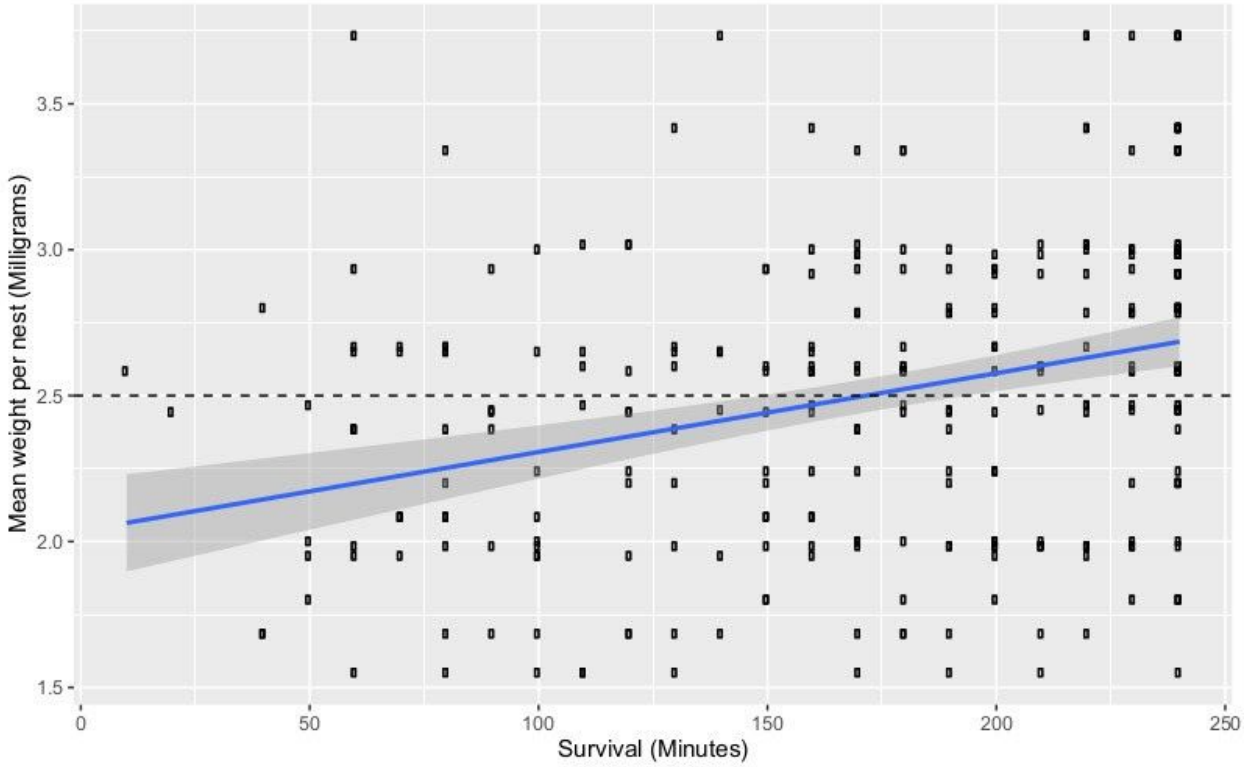

Figure. 10 The relationship between mean dry weight and survival time in the heat-knockdown experiment. Individual survival times and mean dry weights per nest are plotted with black dots. The blue line represents a linear regression between dry weight and survival time, with a 95% confidence interval presented in grey. There was a significant and positive effect of body size, larger workers survived longer in the heat (*p*-value < 0.001, chi-square test, Table 2).

Table 2. Regression tables of the Cox proportional hazards model and mixed effects logistic binomial model from the heat-knockdown resistance analysis. *F. polyctena* is used as a reference category in both models to analyze the effect of the group.

Cox-proportional hazards model							
Dependent: Surv (Minutes, Status)		**Univariable:** Estimate	Hazard ratio (95% CI)	p-value	**Multivariable:** Estimate	Hazard ratio (95% CI)	p-value
Mean_Weight		-0.728	0.483 (0.343-0.679)	< 0.001 ***	-0.558	0.572 (0.373-0.877)	< 0.05 *
Group	pol						
	hyb	-0.320	0.726 (0.399-1.322)	0.295	-0.346	0.708 (0.373 - 0.877)	0.236
	aq	0.550	1.734 (1.001-3.004)	0.050	0.230	1.259 (0.691-2.291)	0.452
Mixed effects logistic binomial model							
Random effect: (1|Population)							
Dependent: Survival ("0","1")		**Univariable:** Estimate	Odds ratio (95% CI)	p-value	**Multivariable:** Estimate	Odds ratio (95% CI)	p-value
Mean_Weight		1.461	4.310 (2.000-9.290)	< 0.001 ***	1.167	3.210 (1.470-7.010)	< 0.01 **
Group	pol						
	hyb	0.636	1.890 (0.803-4.450)	0.145	0.766	2.150 (0.729-6.350)	0.165
	aq	-1.689	0.185 (0.054-0.639)	< 0.01 **	-0.953	0.386 (0.089-1.660)	0.202

3.4 Chill-coma recovery

I predicted that finnish *F. aquilonia* individuals would be more cold-tolerant than *F. polyctena* individuals. Ten ants from each nest were exposed to -13 °C for 25 minutes in the chill-coma recovery experiment. Recovery time from chill-coma ranged from 6.6 to 28 minutes. Ninety-three ants (46 hybrids, 21 *F. aquilonia* and 26 *F. polyctena*) did not wake up during the monitoring time of 30 minutes after cold treatment. Ants from *F. aquilonia* started to recover faster than ants from *F. polyctena* (M±SD = 13.5±0.9 and 16.4±1.1 min for *F. aquilonia* and *F. polyctena*, respectively). According to multiple log-rank tests between the groups, survival rates

differed significantly between ants from finnish *F. aquilonia* and *F. polyctena* (*p*-value < 0.001, log-rank test, Fig. 12). Hybrid ants recovered slower (M±SD = 17.0±0.9 for the hybrids) than *F. aquilonia* (*p*-value < 0.001, log-rank test, Fig.12) but had no difference in survival probability compared to *F. polyctena* (*p*-value = 0.67, log-rank test, Fig.12). Chill-coma recovery experiment was carried out in two consecutive trials, but the batch did not have any effect on the recovery of the individuals (*p*-value = 0.43, log-rank test). There was considerable variability in the survival between populations also in the chill-coma recovery experiment, but as for the heat knockdown resistance experiment, the differences between groups were not driven by a subset of populations (Fig. S2). In the Cox proportional hazards model, mean weight per nest did not have a significant effect on the recovery from cold treatment (*p*-value = 0.112, chi-square test, Table 3). These results were also consistent in the mixed effects logistic binomial model (Table 3).

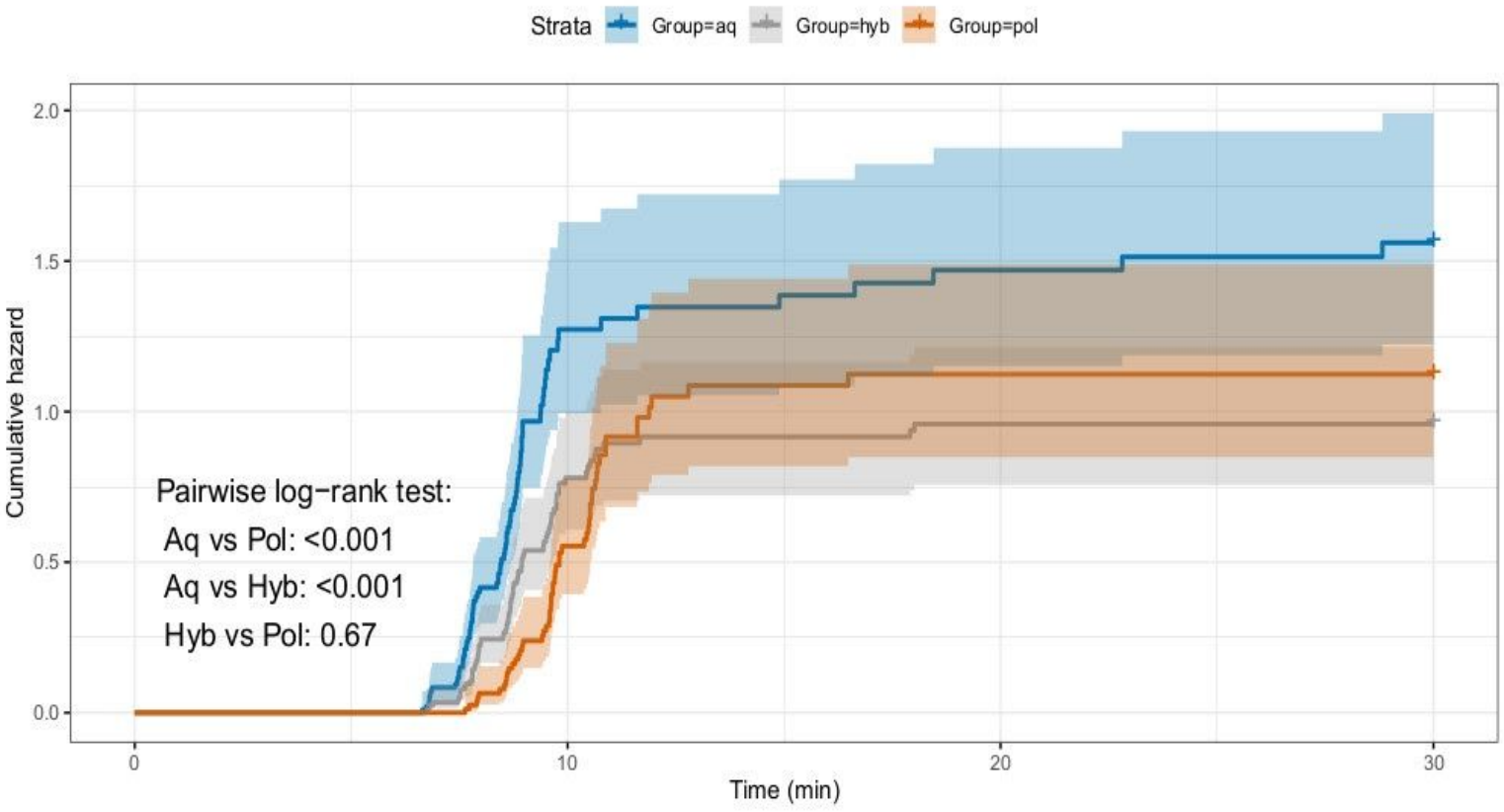

Figure 11. Kaplan-Meier survival curves from the chill-coma recovery experiment. The x-axis indicates the time from the beginning of the experiment when ants are taken out of the cold

chamber and the y-axis the percentage of recovered at certain time points. *F. aquilonia* is indicated with a blue curve, *F. polyctena* in orange and hybrids in grey. The 95% confidence interval of the curve is highlighted with a lighter color. *F. aquilonia* has the steepest curve and individuals start to recover from chill-coma quickest. Hybrids and *F. polyctena* recover slower and there are no significant differences in their recovery.

Table 3. Regression table of the Cox proportional hazards models in the chill-coma recovery analysis. *F. polyctena* is used as a reference category in both models to analyze the effect of the group.

Cox-proportional hazards model

Dependent: Surv (Minutes, Status)		**Univariable:** Estimate Hazard ratio (95% CI)	p-value	**Multivariable:** Estimate Hazard ratio (95% CI)	p-value
Mean_Weight		-0.296 0.744 (0.516-1.071)	0.112	-0.054 0.947 (0.621-1.443)	0.800
Group	pol				
	hyb	0.068 1.070 (0.636-1.799)	0.799	0.069 1.071 (0.634-1.810)	0.796
	aq	0.631 1.879 (1.165-3.030)	< 0.01 **	0.604 1.829 (1.055-3.169)	< 0.05 *

Mixed effects logistic binomial model

Random effect: (1|Population) Dependent: Survival ("0","1")		**Univariable:** Estimate Odds ratio (95% CI)	p-value	**Multivariable:** Estimate Odds ratio (95% CI)	p-value
Mean_Weight		-0.136 0.873 (0.486-1.570)	0.648	-0.001 0.999 (0.544-1.840)	0.997
Group	pol				
	hyb	-0.208 0.812 (0.303-2.180)	0.679	-0.208 0.812 (0.303-2.180)	0.679
	aq	0.510 1.670 (0.460-3.300)	0.318	0.509 1.660 (0.574-4.820)	0.348

4 Discussion

Global surface temperature is increasing at an alarming rate. Local populations can cope with the change, if they have adaptive potential to face the new thermal regime. Hybridization with a closely related lineage is one potential source of adaptive genetic variability. My thesis aimed to investigate thermal adaptation by looking into thermal tolerance differences between two mound-building wood ants *Formica polyctena* and *Formica aquilonia* and their hybrids. My

results showed that parental individuals differed in their thermal tolerance and expressed thermal limits which reflected the two species' distributions, in support to the thermal adaptation hypothesis. The results also showed that hybrids could not combine the thermal tolerance of both parental species as they did not have wider thermal tolerance than parental individuals. Intriguingly, body size had a significant role in thermal tolerance, with bigger ants coping better with higher temperatures.

Formica aquilonia and *Formica polyctena* expressed thermal tolerance limits which reflected their distribution in Europe

My results supported the thermal adaptation hypothesis, as thermal tolerance limits of the finnish *Formica aquilonia* and *Formica polyctena* reflected their current latitudinal range differences. The samples from my thesis represent finnish populations of *F. aquilonia* and *F. polyctena* and could be genetically different from the parental populations in Europe. Especially with *F. polyctena*, it is hard to find samples from Finland that would represent completely the same genetic lineage as in southern Europe. Nevertheless, my results showed that there was a significant difference in thermal tolerance between finnish populations of these species. Finnish *Formica polyctena* was more heat-tolerant and survived longest in the heat-knockdown resistance experiment. Finnish *Formica aquilonia* appeared to be more cold-tolerant and recovered fastest from the chill-coma recovery experiment. Therefore, these two species differ in their thermal tolerance and have thermal tolerance limits that reflect their local surroundings. I tested the differences in thermal tolerance with two thermal assays: heat-knockdown resistance and chill-coma recovery. These assays describe just one aspect of insect thermoregulation, the upper and lower limits, but they have been rigorously tested and shown to characterize thermal tolerance efficiently (Hazell et al. 2008; Jørgensen, Malte & Overgaard 2019; Terblanche et al. 2011).

Thermal tolerance limits are critical in the life cycle of ectothermic organisms. Studies have shown that these limits are often linked to geographic distributions of the species (Andersen et al. 2015; Kellerman et al. 2012; Sunday, Bates & Dulvy 2012). Organisms have

thermal tolerance limits to minimize the cost of thermoregulation by adapting to local surroundings. Most of the research on thermal adaptation of ants is conducted in the tropics, and only few studies test this hypothesis in woodland communities, especially in boreal forests (Garcia-Robledo et al. 2018; Nguyen et al. 2019; Kaspari et al. 2015; Kaspari et al. 2016). The samples in this study were workers, but males and queens could also be included in the experiments to reveal possible differences in thermal tolerance between castes and sexes. In addition to differences between sexes, thermal tolerance may also vary between different life stages, an aspect that is not included in this study. In *Drosophila*, thermal tolerance variations between flies from tropical and temperate regions have been shown in immobile juvenile flies but not in mobile adults (Lockwood, Gupta & Scavotto 2018).

The difference between thermal adaptation and phenotypic plasticity is not always completely unambiguous in thermal tolerance studies (Martin et al. 2019; Sasaki et al. 2019; Yampolski, Schaer & Ebert 2014). Thermal tolerance studies are usually conducted in common garden experiments controlling for the effect of environmental and genetic variation. Unfortunately, common garden experiments are not possible to perform with wood ants. Nonetheless, the populations for my thesis were sampled from approximately the same latitudinal range and originated from a relatively small area around southern Finland and the Åland Islands (Fig. 2). Thus, the differences between finnish populations of these two species are unlikely to originate solely from phenotypic plasticity to different environmental conditions.

Relationship between thermal tolerance and worker body size

Mean dry weight among individuals from the same nest was a significant factor in determining the heat tolerance of the ants. Larger individuals were more heat-tolerant than smaller workers. These results suggest that wood ants with larger body size can tolerate challenging heat conditions better than smaller individuals. Body size differed between groups, finnish *F. aquilonia* was the smallest, hybrids and finnish *F. polyctena* were the biggest. *F. polyctena* and hybrids were also more heat-tolerant than *F. aquilonia*, which could indicate a relationship between large body size and thermal tolerance to high temperatures.

These results are a common finding also in other species across the ant family: upper limits of thermal tolerance often increase with body size (Clémencet et al. 2010; Janowiecki et al. 2020; Ribeiro et al. 2012; Wendt & Verble-Pearson 2016). Thermal tolerance of the workers is crucial for the colony's well-being, since workers are responsible for the mandatory tasks such as cleaning, foraging and taking care of the brood. Larger body size can result in, for example, more effective desiccation tolerance and cuticular thermal resistance (Cerdá 1997; Galushko et al. 2005). Bigger workers can go out of the nest on longer foraging trips even when the outside temperature is high and defend the nest more efficiently (Andersen et al. 2015; Shik 2019).

I did not find a clear effect of body size on cold tolerance. This could be due to methodological issues, since mean weight per nest was calculated from the heat-knockdown experiment samples. Still, there were clear significant weight differences between groups of finnish *F. aquilonia*, *F. polyctena* and hybrids in these samples. A similar response of body size being linked to upper limits, but not lower limits of thermal tolerance has been detected in, for example, army ants (Baudier & O'Donnell 2018). Generally, the relationship between the lower limits of thermal tolerance and body size in insects is not as well documented as upper limits. Conflicting studies have revealed smaller body size enhancing cold-tolerance as well as larger size, some have found no interaction at all (Angilletta et al. 2007; Hahn et al. 2008; Modlmeier et al. 2012). This can indicate that the molecular mechanisms involved in insect cold-tolerance are not so tightly linked to body size than in heat-tolerance. The results from my thesis are consistent with this hypothesis, but additional experiments are needed to provide a clear answer.

Thermal tolerance in hybrids

Finnish *Formica polyctena* x *Formica aquilonia* hybrids expressed highly similar upper limits of thermal tolerance to finnish *F. polyctena*. On the other hand, the hybrids expressed low tolerance to cold and recovered poorly from the chill-coma experiment. Therefore, the hybrids did not have wider thermal tolerance than finnish parental species even though they have

alleles from both heat-tolerant *F. polyctena* and cold-tolerant *F. aquilonia*. Overall, the hybrids expressed thermal limits that were more similar to finnish *F. polyctena* than to *F. aquilonia*. Also in the genomic analysis, the hybrids appeared to be genetically more similar to *F. polyctena* than to *F. aquilonia* (Fig. 6, Fig. 7). As mentioned above, it is challenging to find samples from pure
F. polyctena in Finland, and the nests in my study could represent more admixed *F. polyctena* than populations in central Europe. Thus, the similarity in thermal tolerance between the hybrids and *F. polyctena* could result from similarities in their genetic background, i.e., that finnish *F. polyctena* individuals sampled for our study may be genetically more distant to *F. polyctena* than finnish *F. aquilonia* individuals are to *F. aquilonia*.

The hybrids had surprisingly poor cold tolerance even though they have alleles from cold-tolerant finnish *F. aquilonia*. Generally, hybrid offspring can suffer from genetic incompatibilities which can be witnessed in hybrid breakdown and reduced fitness (Albrechtova et al. 2012; Kenchington 2019). Insect cold-tolerance has been linked to the expression of heat shock proteins (Rinehart et al. 2007). These proteins are an essential factor for thermal tolerance, and their dysfunction has been shown to disrupt insect chill-coma recovery and the repairment of chill injury (Colinet, Lee & Hoffman 2010; Kostal & Tollarova-Borovanska 2009). Considering the results from the chill-coma experiment, the hybrids could have non-functioning pathways in the heat-shock proteins due to their admixed genomes. This hypothesis could have to be tested thoroughly, for instance by documenting heat-shock proteins expression in extreme temperatures in hybrids and parental species.

Thermal tolerance of hybrids has been tested across taxa and outcomes vary substantially (Culumber et al. 2012; Lockwood, Gupta & Scavotto 2018; Martins et al. 2019; Wells et al. 2016). Hybrids of two kelps *Laminaria digitata* and *Laminaria pallida* express hybrid vigour and outperform both parental species in upper limits of thermal tolerance (Martins 2019). On the other hand, hybrid sea urchin offspring have shown to be inferior to parental species in their thermal tolerance (Lamare et al. 2018). But in brook trouts, for example, no significant difference between hybrids and parental species could be found (Wells et al. 2016). Lastly, there is also evidence of hybrid individuals expressing novel phenotypes by increased

thermal tolerance compared to parental species (Pereira, Barreto & Burton 2014). The results from my thesis provided insights to the possible outcomes of hybridization, but deeper knowledge on the adaptive potential of these hybrid ants requires research on the genetic basis of thermal adaptation and how temperature affects the populations on a long-term scale.

Future directions for the populations of *Formica aquilonia*, *Formica polyctena* and their hybrids in the Northern Europe

The results from my thesis showed that finnish *F. aquilonia* and *F. polyctena* differ in their thermal tolerances and these differences in thermal limits reflect their current distributions in Europe. The hybrids of these wood ant species had highly similar upper limits of thermal tolerance than finnish *F. polyctena* but surprisingly poor cold-tolerance and therefore did not express wider thermal tolerance than the parental species.

In the future, the thermal regime of Northern Europe is likely to shift towards increasing overall temperatures and snowless winters (Füssel et al. 2017; Kovats et al. 2014). Periods of intense heat extremes are likely to increase and extreme cold periods become less frequent (Coumou & Robinson 2013; Lorenz, Stalhandske & Fischer 2019). Change in these thermal conditions will expand species ranges poleward and to higher elevations, which has already been witnessed globally (Chen et al. 2011; Crickenberger & Wethey 2018; Hickling et al. 2006; Pinsky et al. 2013). Heat-tolerant species can expand their range northwards and replace the native species (Calosi, Bilton & Spicer 2008; Hill, Chown and Hoffman 2013). Considering the two wood ant species, heat-tolerant *F. polyctena* may possibly extend its range northwards in the future, and perhaps outcompete *F. aquilonia* in some regions. *F. aquilonia* has already restricted distribution and cannot expand northwards in Europe. Replacement by more heat-tolerant species, which has resulted in range retraction of the more heat-sensitive species has been witnessed in other insects as well (Birkett, Blackburn & Menéndez 2018; Calosi, Bilton & Spicer 2008).

Hybridization in nature is likely to become more frequent when climate change affects species' range boundaries and new contact zones appear (Chunco 2014, Sánchez-Guillén et al.

2016). Already in the past, hybridization has enabled multiple species to expand their ecological niche towards new habitats when climate has altered rapidly (Keller et al. 2013, Nolte et al. 2005, Papadopulos 2013). This type of range expansion fueled by hybridization has been witnessed in, for example, the Lake Malawi cichlid fish that have adapted to different niches in the lake system and diversified into multiple species from an ancestral hybrid population (Keller et al. 2013, Svardal et al. 2020). Hybridization between heat-tolerant *F. polyctena* and cold-tolerant
F. aquilonia can further aid the poleward range expansion of *F. polyctena* when new contact zones and ecological opportunities appear.

Hybridization between *F. aquilonia* and *F. polyctena* in southern Finland has been the subject of wide and intensive research over the years (Beresford et al. 2017; Kulmuni & Pamilo 2014; Kulmuni, Seifert & Pamilo 2010; Martin-Roy & Kulmuni 2019). The hybrid populations are shown to be stable and maintained throughout the years (Kulmuni, Seifert & Pamilo 2010). There is also evidence that the parental-like allele frequency fluctuates between the years in the hybrid males, and that these fluctuations correlate with early spring temperatures (Martin-Roy & Kulmuni 2019). The frequency of the finnish *F. polyctena* alleles in hybrid males was higher in warmer years while the frequency of the finnish *F. aquilonia* alleles was higher in colder years (Martin-Roy & Kulmuni 2019). Combined with the results from my thesis, this suggests that the potentially adaptive heat- and cold-tolerant alleles from the finnish parental species could help the hybrids to cope with fluctuating temperatures.

Conclusions

This thesis combined population genomic tools and temperature assays to study the thermal tolerance differences in two mound-building wood ants and their hybrids in Finland. My results showed that the finnish populations of two parental species *Formica aquilonia* and *Formica polyctena* have different limits of thermal tolerance which could suggest that they are adapted to a thermal regime that reflects their distributions in Europe. On the other hand, the hybrids of these two species did not express wider thermal tolerance than parental species. These results

contribute to building up the knowledge on the outcomes of hybridization and the potential that species possess in coping with the environmental change. Wood ants are keystone species in the boreal forests, and threatened by increasing global temperatures and habitat destruction. The findings of my thesis shed a light on population dynamic changes for these species in the face of global climate change.

5 Acknowledgements

Special thanks to my supervisors Jonna Kulmuni and Pierre Nouhaud for their tireless support and help throughout this project. Thanks should also go to the Societas pro Fauna et Flora and research group SpecIAnt for providing funding that allowed me to carry out this thesis. I wish to thank all members of the research group SpecIAnt for continuous support and encouragement. I would like to especially thank Raphaël Martin-Roy for his effort in conducting the field experiments and mentoring in the laboratory. Thanks also to the multi-disciplinary members of the Evolution, Sociality and Behavior research group for the feedback and welcoming research environment. I would like to thank Tvärminne Zoological Station for enabling the field experiments and providing great research facilities as well as the staff in Molecular Ecology and Systematics - laboratory for their guidance and facilities.

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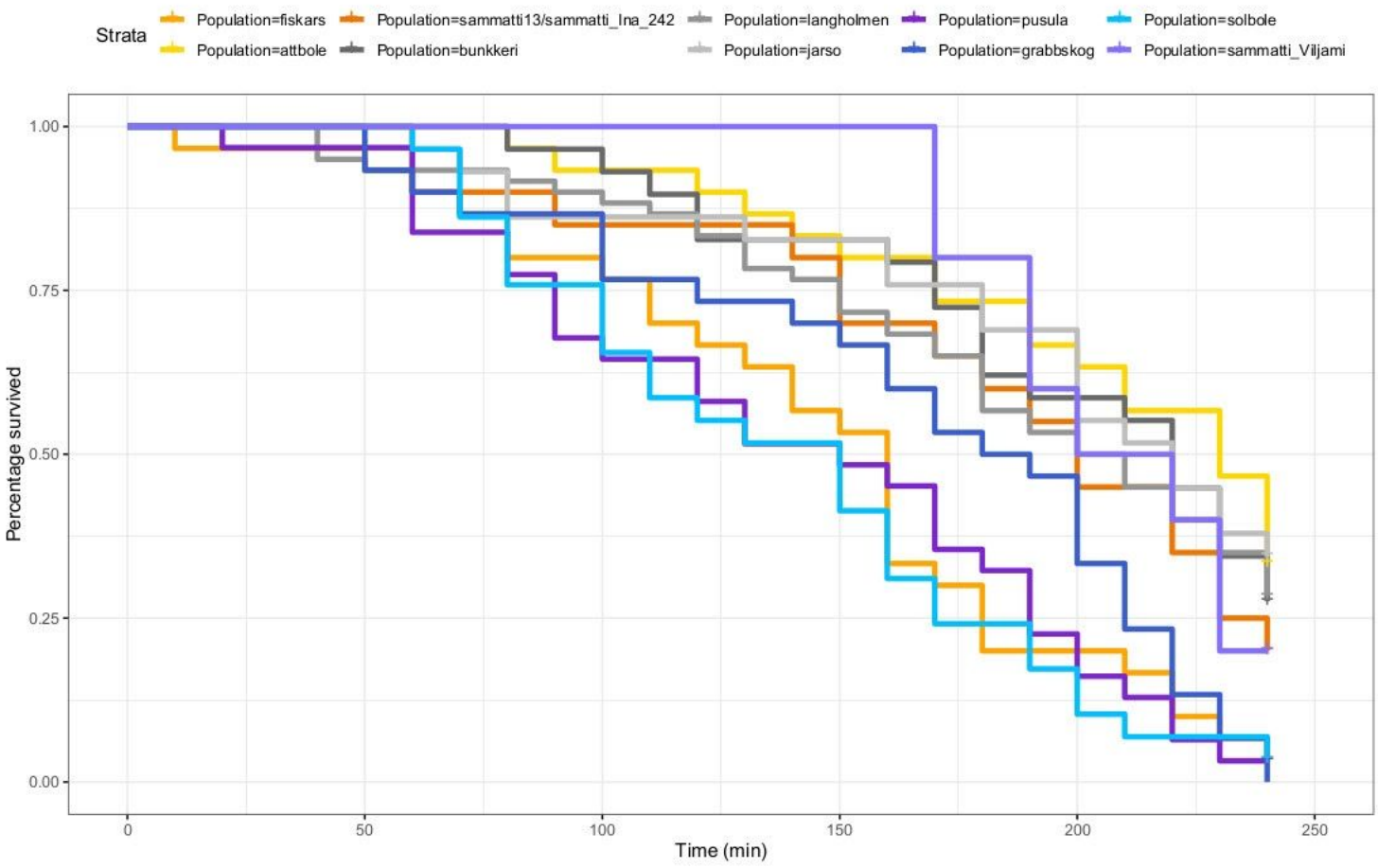
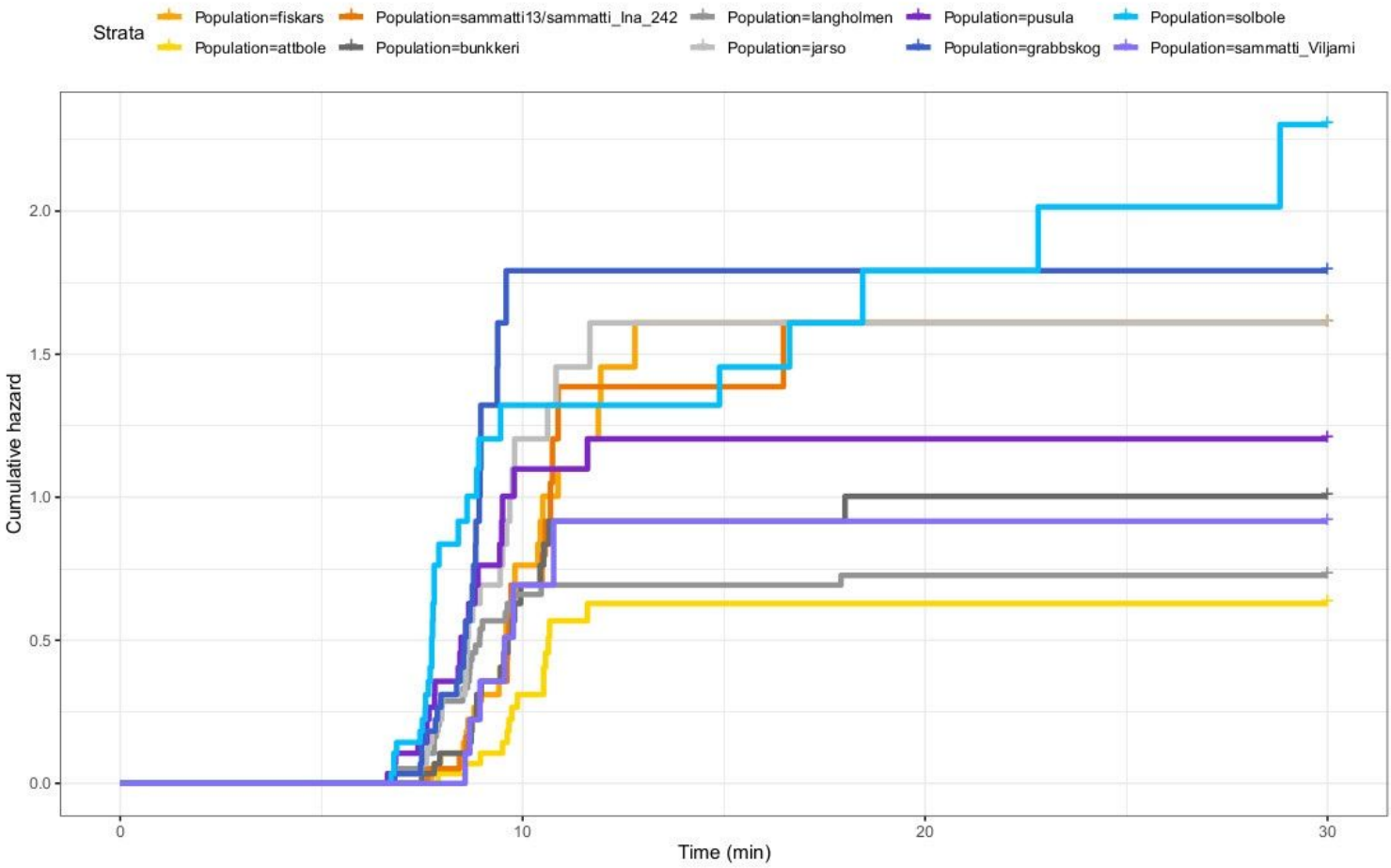
Appendices

Supplementary figures



Supplementary figure 1. Kaplan-Meier survival curves per population from the heat-knockdown experiment. X-axis indicates the time from the beginning of the experiment and y-axis the percentage of individuals alive at certain time points. Finnish *F. aquilonia* populations are indicated with blue lines, *F. polyctena* in orange and hybrids in grey.



Supplementary figure 2. Kaplan-Meier survival curves per population from the chill-coma recovery experiment. X-axis indicates the time from the beginning of the experiment when ants are taken out of the freezer and y-axis the percentage of recovered at certain time points. Finnish *F. aquilonia* populations are indicated with blue lines, *F. polyctena* in orange and hybrids in grey.