Table des matières

Liste des figures

Liste des tableaux

Chapitre 1. Introduction

Les mitochondries sont des organites essentiels dans les processus métaboliques fournissant de l'énergie, sous forme d'ATP, à la cellule. Il est maintenant communément admis que les mitochondries descendent d'α-protéobactéries endosymbiotiques (Gray *et al.*, 1999; Lang *et al.*, 1999a; Lang *et al.*, 1999b; Kurland and Andersson, 2000). Ce sont des organites semi-autonomes possédant leur propre matériel génétique (ADN mitochondrial) ainsi que la machinerie pour sa réplication, sa transcription et sa synthèse protéique. Elles auraient divergé des bactéries il y a environ 1,5 à 2 milliards d'années (Sicheritz-Ponten *et al.*, 1998), présentant désormais plusieurs caractéristiques d'une adaptation à la vie endosymbiotique. Depuis leur incorporation dans les cellules, elles ont évolué selon deux modes distincts. Le premier est un mode réductif qui consiste en une diminution du nombre de produits synthétisés par l'organite permettant une protection accrue de l'information génétique ainsi transférée puisque le milieu nucléaire est moins riche en radicaux libres qui sont reconnus comme agents mutagènes (Allen, 1993; Allen and Raven, 1996) et qu'il présente des mécanismes de réparation de l'ADN, mécanismes absents chez les mitochondries (Scheffler, 1999). Ainsi, il se produit un remplacement des produits synthétisés par la mitochondrie par des produits synthétisés via le génome nucléaire. Ceci a comme conséquence que les gènes non essentiels à une survie intracellulaire peuvent être perdus par la mitochondrie. La sélection purificatrice sur ces gènes est alors suspendue permettant la délétion (ou l'inactivation) de ces derniers lors de mutations (Kimura, 1992). En parallèle à ce phénomène, les gènes mitochondriaux uniques et essentiels peuvent être transférés au génome nucléaire si les produits synthétisés sont importables depuis le cytosol (Gray *et al.*, 1999; Lang *et al.*, 1999a; Berg and Kurland, 2000; Kurland and Andersson, 2000). Des peptides d'origine nucléaire ont été ainsi

identifiés chez des mitochondries, suggérant une forme de transfert en sens inverse, soit du noyau vers l'organite (Lang *et al.*, 1999a). Ce deuxième mode d'évolution est appelé extensif, et permet à l'organite de bénéficier de l'évolution nucléaire. Donc, la majorité des gènes codés initialement par la bactérie ancestrale ayant disparu du génome mitochondrial (Lang *et al.*, 1999a; Kurland and Andersson, 2000) les mitochondries des vertébrés présentent actuellement un génome réduit codant uniquement pour des protéines impliquées dans la production énergétique (phosphorylation oxydative), vingt-deux ARN de transport et deux ARN ribosomaux (Scheffler, 1999; Blier *et al.*, 2001). Ce transfert d'information génétique implique un niveau élevé d'interaction entre les deux génomes, compte tenu que des protéines codées par différents génomes doivent dorénavant fonctionner ensemble. En effet, afin de mener à bien leurs fonctions métaboliques, les mitochondries nécessitent l'expression des centaines de gènes codés par le génome cellulaire et 37 codés par l'organite (Sackton *et al.*, 2003). Donc, le fitness et les propriétés fonctionnelles dépendent de l'interaction de protéines codées par les deux génomes.

Les mitochondries assurent de nombreuses fonctions vitales au sein de la cellule à l'aide de protéines d'origine nucléaire, comme la production d'énergie sous forme d'ATP. Elles sont le siège de plusieurs voies métaboliques (par exemple : le cycle de Krebs, la β-oxydation des acides gras) mais seule la phosphorylation oxydative implique des protéines codées par le génome mitochondrial. La phosphorylation oxydative est le dernier stade de la respiration cellulaire, toutes les autres voies métaboliques y menant (Lehninger *et al.*, 1994). Située dans la membrane interne des mitochondries, la chaîne respiratoire consiste

en une série de transporteurs d'électrons. Ces électrons sont utilisés comme source d'énergie pour la phosphorylation de l'ADP en ATP (molécule hautement énergétique), réaction qui transforme une molécule d'oxygène (O_2) en 2 molécules d'eau (2 H_2O). Cette chaîne respiratoire est constituée de quatre complexes : la NADH déshydrogénase (complexe I), la succinate déshydrogénase (complexe II), la cytochrome c réductase (complexe III) et la cytochrome c oxydase (complexe IV). Un cinquième complexe ne faisant pas partie de cette chaîne, l'ATP synthase, utilise les protons transportés *via* cette chaîne pour phosphoryler l'ADP. Le complexe IV est constitué de treize sous-unités dont dix codées par le génome nucléaire. Celles-ci remplissent surtout des fonctions structurelles alors que les trois sous-unités contenant les centres catalytiques (COX I, II, et III) sont codées par le génome mitochondrial. Ce complexe est reconnu comme jouant un rôle important dans la régulation de la respiration mitochondriale (Blier and Lemieux, 2001). Alors que les électrons sont tranférés entre les complexes II et III par l'ubiquinone, ils passent du complexe III au complexe IV *via* une protéine codée par le génome nucléaire : le cytochrome c.

Plusieurs études ont montré que le métabolisme de la chaîne respiratoire est hautement sensible à la température, surtout chez les animaux ne contrôlant pas leur température corporelle. Les espèces de zones froides présentent généralement des capacités catalytiques supérieures à celles de zones chaudes (Hochachka and Somero, 2002), lorsque mesuré à la même température. Des analyses d'activités enzymatiques (lactate déshydrogénase et citrate synthase) menées sur des ectothermes de régimes thermiques divers ont montré des activités deux à trois fois supérieures chez les espèces acclimatées aux milieux froids

lorsque mesurées aux mêmes températures expérimentales (Hochachka and Somero, 2002). De plus, des analyses des constantes d'efficacité catalytique (Kcat), une mesure du taux de substrat converti en produit par site actif par unité de temps, ont aussi montré des taux deux à trois fois supérieurs chez les espèces adaptées aux milieux froids. Cette diminution de la capacité catalytique des enzymes en fonction de la température peut être expliquée par un plus grand besoin de stabilité au niveau de la structure protéique à haute température. Ainsi, compte tenu que l'activité enzymatique est fonction de la constante d'efficacité catalytique et de la concentration en enzyme, les mécanismes de compensation enzymatique, à l'échelle évolutive, peuvent être à la fois attribuables à des ajustements des capacités catalytiques par des substitutions d'acides aminés ou à des ajustements des taux de synthèse des protéines (Hochachka and Somero, 2002).

Lorsqu'une enzyme réagit avec son substrat, il s'ensuit un changement de conformation au niveau structurel de l'enzyme. Ce changement de conformation nécessite une certaine flexibilité de la structure, et est donc négativement lié à la stabilité de celle-ci (Hochachka and Somero, 2002). La structure est maintenue par des liaisons hydrogènes entre les éléments chimiques présents au sein des protéines, mais cette stabilisation structurelle diminue aussi l'efficacité enzymatique (Hochachka and Somero, 2002). Il a été démontré qu'il existe une forte corrélation entre la température corporelle et la stabilité protéique (Hochachka and Somero, 2002).

Il semble donc assez probable que des espèces vivant dans des environnements thermiques différents possèdent des enzymes aux propriétés adaptées aux gammes de températures

rencontrées pour la catalyse et la régulation enzymatique propres à chacune des espèces. Ainsi, on peut s'attendre à ce que la température corporelle, et non la distance phylogénétique soit le facteur déterminant expliquant les différences d'efficacité d'une enzyme.

L'héritabilité maternelle des mitochondries, chez la majorité des espèces, permet de facilement modifier et étudier les interactions entre le génome mitochondrial et nucléaire; par rétro-croisement répété ou hybridation, il est possible d'en étudier l'impact sur la valeur reproductive, le taux de survie, le taux de respiration, d'organismes et sur les propriétés fonctionnelles de cellules modifiées et de mitochondries. Par exemple, Burton et ses collaborateurs (Edmands and Burton, 1998; Burton *et al.*, 1999; Edmands and Burton, 1999; Willett and Burton, 2001; Rawson and Burton, 2002), dans des études récentes, ont montré que les interactions nucléaire-mitochondriales sont responsables de différences observables dans l'activité de la COX et du taux de survie suite à une hybridation entre des populations de copépodes. De plus, Schmidt *et al.* (2001) ont montré que les résidus protéiques, originaire du noyau, physiquement plus proches de résidus codés par l'organite évoluent plus lentement que ceux étant physiquement éloignés, ce qui suggère la présence d'une sélection contraignante. L'étude des interactions suggère également l'existence d'un point de non-retour dans l'évolution des deux génomes. En effet, Barrientos *et al.*, (2000) ont réussi à restaurer le métabolisme oxidatif dans des cellules dépourvues de mitochondries dans lesquelles des mitochondries d'espèces apparentées ont été introduites. Le même résultat ne put être obtenu avec des mitochondries d'espèces éloignées.

Afin d'étudier le rôle de la sélection naturelle sur l'évolution de l'ADN et la co-évolution de complexes protéiques, deux voies non-exclusives peuvent être étudiées. Traditionnellement, les études se sont basées sur des analyses génétiques. Ce premier type d'analyse est fondé sur les variations au niveau génotypique, sans considérer leurs influences au niveau phénotypique (physiologie et valeur reproductive). Ainsi, deux espèces éloignées phylogénétiquement présentent un nombre élevé de substitutions au sein desquelles les mutations importantes, ou les remplacements, présentant des différences significatives ne pourront être détectées. Une seconde option est d'étudier les conséquences des substitutions génétiques au niveau physiologique. Plusieurs études ont, par le passé, porté sur l'étude des propriétés fonctionnelles des enzymes dans un cadre de co-évolution et ont principalement portés sur la mesure de la valeur reproductive des organismes (Hutter and Rand, 1995; Rand *et al.*, 2001) et la mesure d'activités enzymatiques (Kenyon and Moraes, 1997; Burton *et al.*, 1999; Rawson and Burton, 2002). Holland *et al.*, (1997) ont démontré que chez les poissons du genre *Sphyraena*, la substitution d'un seul acide aminé, causée par la substitution non-synonyme d'un nucléotide, suffisait pour assurer une compensation partielle des propriétés fonctionnelles des enzymes à différentes températures. Ainsi, une telle substitution serait donc sans signification génétique (non-détectée lors d'analyses de séquençage), bien que démontrant une grande importance physiologique.

Le présent projet vise à étudier l'influence de la température comme contrainte sélective importante sur les propriétés fonctionnelles des complexes cytochrome c oxydase et cytochrome c. Les interactions de protéines d'origine mitochondriale et nucléaire au sein

de la chaîne respiratoire fournissent une excellente opportunité d'étudier l'impact de la température sur la coévolution des génomes mitochondriaux et nucléaire. Ainsi, quatre espèces de deux régimes thermiques (froid : vers de mer (*Neanthes virens)* et omble chevalier (*Salvelinus alpinus*) et chaud (lapin et abeille (*Apis mellifera*)) ont été sélectionnées et les propriétés fonctionnelles de la COX ont été mesurées à trois températures (10, 20 et 30°C) en présence de deux types de cytochrome c (CYC) (levure (*Saccharomyces cerevisae*) et cœur de cheval) alternativement. Dans une étude similaire portant sur des populations de copépodes, Rawson and Burton, (2002), mirent en évidence des impacts indépendants de l'origine de la COX, de l'origine de la CYC et de la température sur l'activité maximale de la COX. Compte tenu qu'il est communément accepté que la température influence la fluidité membranaire (Hazel, 1995), la solubilité des gaz (Hochachka and Somero, 2002) et la diffusion des petites molécules à travers le cytosol (Sidell and Hazel, 2002) et que la COX utilisée dans la présente étude soit issue d'organismes ayant évolués dans des régimes thermiques différents, il est possible de supposer obtenir des résultats similaires pour les espèces de régimes thermiques semblables.

Si la température n'est pas (ou ne fut pas) une pression sélective primordiale pour l'évolution de cette enzyme, il est possible de supposer que les mutations génétiques se soient fixées aléatoirement dans les génomes des espèces étudiées. Les hypothèses actuelles qui tentent d'expliquer l'évolution mitochondriale sont basées sur le concept de neutralité ou quasi-neutralité des mutations et considèrent que la plupart des mutations fixées dans un génome présent un avantage nul (ou quasi-nul) sur le phénotype sauvage

alors que la majorité des mutations subdélétères ou délétères sont éliminées. Ainsi, comme les espèces étudiées sont génétiquement indépendantes, nous pouvons postuler que le nombre de mutations différentes fixées peut être corrélé au temps de séparation entre deux espèces. Deux espèces distantes devraient ainsi présenter un nombre plus élevé de mutations non-partagées que deux espèces rapprochées. Un gradient devrait donc être observée entre les diverses espèces étudiées ici, allant de l'abeille au lapin (abeille, *N.virens,* omble chevalier et finalement lapin). Par contre, si la température joue (ou a joué), l'évolution devrait (ou a dû) favoriser des adaptations permettant le maintien des propriétés fonctionnelles et de la régulation des mécanismes aux différentes températures auxquelles chaque organisme est acclimaté. Compte tenu qu'il a déjà été démontré que la vitesse maximale de la COX est très influencée par la température (Blier and Lemieux, 2001) et que cette enzyme joue un rôle-clé dans le contrôle du métabolisme mitochondrial, nous croyons qu'elle a été soumise à une forme de sélection positive, présentant des adaptations aux diverses températures d'acclimatation. Les mutations ne seraient donc pas fixées aléatoirement mais plutôt sélectionnées positivement (ou négativement). Dans cette hypothèse, aucun gradient ne serait détectable et les organismes de régimes thermiques semblables présenteraient des propriétés cinétiques semblables. Ainsi, la température environnementale et corporelle, plutôt que le statut phylogénétique seraient des facteurs déterminants des propriétés fonctionnelles des enzymes (Km, Vmax et thermosensibilité).

Chapitre 2.

DOES CYTOCHROME C SET THE THERMAL SENSITIVITY OF CYTOCHROME C OXIDASE?

B.A. AUCLAIR[1] ET P.U. BLIER[1]

[1]Département de Biologie, Université du Québec à Rimouski, 300 Allée des Ursulines

Rimouski (Québec) Canada, G5L 3A1

Corresponding Author : Tel : 1 (418) 723-1986 ext 1852 Fax 1 (418) 724-1849

e-mail address: Pierre_blier@uqar.qc.ca

1. Abstract

In the present study, we monitored the functional properties (Vmax and Km) of a key-enzyme of the mitochondrial metabolism, the cytochrome c oxidase. A partial compensation of the Vmax and a conservation of the affinity of the enzyme for its substrate were detected. The affinity of the enzyme for its substrate seems to have been more protected than the Vmax. The species of origin of the substrate also had a significant impact on the functional properties ($p<0,05$; anova) and was responsible for an important part of observed differences when species adapted to similar thermal environment are studied, no matter the phylogenetic distance between them. Therefore, the temperature of adaptation, rather than the phylogenetic distance, appears as a good predictor of the functional properties of species. Thus temperature has been an important evolutionary drive, forcing the adaptation of species to different environment. In a second experiment, we showed that the use of spectrophotometry to measure the activity of cytochrome c oxidase (COX) under-estimate the value of the Vmax by 25 to 92,25% depending of species and substrate concentration.

2. Introduction

It is commonly accepted that mitochondria originated from symbiotic α-proteobacteria and have since evolved toward an strict endo-symbiotic life. It seems to have lost an important part of its genome, some of which is now found in the nucleus. Several examples of gene transfer between the nucleus and the organelle have been discovered and it is now accepted that mitochondria present a reduced genome interacting with proteins coded by the cell nucleus (Blier *et al.*, 2001). This is mostly attributed to a better protection of the nucleus genome , i.e. a lesser presence of free radicals (Allen and Raven, 1996) and a presence of a transcription reparation mechanism in the latter (Lang *et al.*, 1999a). This transfer of genetic information implies a high level of interaction between the two genomes, as proteins from different origin have to work together. Indeed, in most species, mitochondrial function requires the expression of hundred of nuclear-encoded genes and 37 mitochondrial genes (Sackton *et al.*, 2003). Mitochondria are the site of several important metabolic pathways, but among those only the oxidative phosphorylation implies proteins coded by both the nucleus and the organelle genome. COX is a complex of 13 subunits, 10 encoded by the nucleus, assuring mainly structural function, and 3 active sites encoded by the mitochondrion. Thus, fitness and performance traits are dependant on the interaction of the proteins coded by the two genomes. The maternal inheritance of mitochondria, in most species, allows easy modifications and studies of the interactions between the nucleus and the organelle: by repeated back-crossing or hybridization, it has been possible to study the impact of manipulations of nuclear environment for a given mitochondrial genome on fitness, survival rate, respiration rates, of organism or functional properties of manipulated

cells or mitochondria. For example, in recent studies, Burton and co-workers (Edmands and Burton, 1998; Burton *et al.*, 1999; Edmands and Burton, 1999; Willett and Burton, 2001; Rawson and Burton, 2002) have shown that nuclear-mitochondrial interactions were responsible for the variations of COX activity and survival of organisms after hybridization of different populations of copepods from the coast of California. Furthermore, oxidative respiration has been restored in human mitochondria-free cells in which mitochondria from close-related species were introduced, but not with distant species (Barrientos *et al.*, 2000), suggesting a threshold effect of nuclear-mitochondrial disruption. Finally, Schmidt *et al.*, (2001), showed that protein residues originating from nucleus and physically closer to residues coded by mitochondrion genome evolved slower than other nuclear encoded residues therefore suggesting adaptations of structurally linked residues from nuclear and mitochondrial genome.

In order to assess how co-evolution has taken place, many studies have used fitness essays (Hutter and Rand, 1995; Rand *et al.*, 2001), enzyme activities (Kenyon and Moraes, 1997; Burton *et al.*, 1999; Rawson and Burton, 2002) or sequence alignment (Schmidt and Shaw, 2001; Schmidt *et al.*, 2001). The latter are based on genotypic analysis and often do not imply the phenotypic impact (physiology and fitness) of the differences observed. Furthermore, genetic analyses have a major drawback; they are based on statistical analysis to determine the significant differences between the sequences studied. Two sequences from animals phylogenetically distant would show a high number of substitutions without emphasizing important replacements or mutations. Studies on cytoplasmic enzymes implicated in the energetic metabolism have shown that the substitution of only one amino

acid is enough to modify the kinetic properties of the enzyme. For example, when working on two populations of the genus *Sphyraena,* Holland *et al.*, (1997) showed that only two changes in the sequence of amino acids could modify the Km or thermal stability of lactate dehydrogenase (LDH). Therefore, it appears that the measurement of the enzyme activity alone is not sufficient to efficiently detect functional adjustment of enzymes.

Cold-adapted species may possess adaptations to counter the effect of low temperature on physiological reactions. They often express higher enzyme activity at low temperature and in high viscosity medium which could be caused by two majors process modifications: 1) an increase in the concentration of enzyme or 2) a higher catalytic efficiency of the enzyme (Hochachka and Somero, 2002). For example, Hochachka and Somero, (2002), showed that thermal compensation can be obtained via a modification of the catalytic efficiency in LDH. However, this compensation is rarely complete and has usually been shown to be partial in most species (Zecchinon *et al.*, 2001; Hochachka and Somero, 2002).

In order to test the impact of temperature as a selective pressure on the kinetic properties of the COX complex, four species from two thermal regimes (cold: *Neanthe. virens* and arctic charr (*Salvelinus alpinus)* and warm: honeybee (*Apis mellifera)* and rabbit (*Oryctolagus cuniculus*), were selected. The Km and Vmax of the enzyme were measured at three temperatures (10, 20 and 30°C) in presence of alternatively two types of cytochrome c (yeast (*Saccharomyces cerevisae)* and horse heart (HH)). Rawson and Burton, (2002), in a similar experiment on copepods have found independent impact of temperature, origin of

COX and origin of CYC (, but not for the interaction of the three on the maximal activity of COX. Considering that temperature affects membrane fluidity (Hazel, 1995), gas solubility (Hochachka and Somero, 2002) and diffusion of small molecules through the cytosol (Sidell and Hazel, 2002) and the fact that COX in the present study are from species evolving at two thermal regimes (cold adapted and warm adapted species), we expect to find similar results for species living within the same regimes. If temperature is not a determinant selective pressure on this enzyme, we could suggest that fixed-mutation related to the time of separation between two species would be the major parameter in the determination of kinetic parameters when we use common source of cytochrome c. The kinetic parameter should, in fact be strongly affected by the divergence time between the species from which cytochrome c oxidase has been extracted and the organism from which cytochrome c has been purified. However, as COX Vmax has been shown to be strongly affected by temperature (Blier and Lemieux, 2001) and that it is an important enzyme in the control of the mitochondrial metabolism, we believe that evolution has favoured adaptations allowing the maintenance of the functional properties and the regulation mechanisms at different temperatures to which organisms are exposed. Therefore mutations would not be only randomly fixed, but some should also be positively selected. In such case, organisms from similar thermal environment would show similar kinetic properties.

3. Material and Methods

Animal and chemical supply

Two species of ectotherms and two species of endotherms were used for this experiment. Specimens of *N. virens* were captured in March 2003 in Sainte-Luce sur Mer, Québec. Arctic charr were bought from the Les Cèdres fishfarm, Luceville. Rabbits were bought from a local farm and specimens of honeybees were obtained from a local bee-keeper; les Productions du Vieux Moulin, Sainte-Luce sur Mer in July 2003.

Chemicals were bought from Sigma-Aldrich corporation.

COX extraction

All extractions were done with fresh tissue. For *N. virens* and honeybees, individuals have been pooled to obtain enough purified enzymes to allow all essays. A total of about 25 and 100 individuals for each extraction were respectively pooled prior to homogeneization. Four extractions were done on each species. For arctic char, both lateral-red muscles were removed and pooled. Muscles from the rear-upper leg were removed and pooled for rabbits.

Once removed, tissues were homogenized in 4 volumes of an homogeneization buffer (250 mM sucrose; 1 mM EDTA; 30 mM HEPES; 1% (w/v) bovine albumin serum and 0,1% of tween 20; pH 7,4) using a tekmar polytron. All procedures were performed on ice, except

the centrifugations which were done at 4°C. The homogenate was then centrifuged (Heraeus Sepatech; suprafuge 22) at 750g for 10 minutes and the supernatant, without the layer of lipid at the surface, was removed and kept at 4°C until further use. The pellet was resuspended in 3 volumes of homogeneization buffer and re-homogenized with a tekmar polytron. A second centrifugation at 750*g* x 10 min was done. The supernatant was then removed and mixed with the previous one. They were then centrifuged at 27 000*g* for 10 minutes and the supernatant, containing the cytochrome c, was discarded. The pellet was resuspended in 3 volumes of Tris-HCl buffer (10 mM; pH = 7,4) containing 0,1% of tween-20. A second centrifugation of 27 000*g* x 10 minutes was then done. The pellet was then resuspended in 4 volumes of HEPES-KOH buffer (100mM; pH = 7,4) and frozen in 500ul aliquots in liquid nitrogen.

Respiration measurements

Activity of COX was measured in a polarographic system, using an oxymeter (Cameron Instrument Co., Tx, USA), two Clark-type O_2 electrodes and two water-jacketed glass chambers. The system was calibrated using distilled water; 637 nmol O_2 ml^{-1} at 10°C, 521 nmol O_2 ml^{-1} at 20°C and 444 nmol O_2 ml^{-1} at 30°C (Reynafarje *et al.*, 1985). The assays were conducted in 130 mM KCl, 30 mM HEPES, 10 mM KH_2PO_4, 11 mM $MgCl_2$, 20 mM glucose, 0,5% BSA and 10mM ascorbic acid pH 7,4 (Blier and Lemieux, 2001). Residual activity was measured for 3 minutes and the respiration was initiated by the addition of CYC to reach desired concentration every 2 minutes. Respiration rates were determined by increasing the concentration of CYC in the reactive chambers, ranging from 2 to 480 µM of

CYC. A first reaction was monitored for low concentrations of CYC, from 2 to 30 µM. The same protocol was used subsequently, for higher concentrations of CYC (30 to 480 µM). This was used to maximize the readability of the activity at low concentrations of CYC. All assays were done on four extractions for each species. Under these conditions, reaction rates for each concentration were linear and were calculated using the best linear fit to the 1,5 min trajectories. Reaction rates were normalized to protein concentration of the homogenates as determined with bicinchoninic acid reagent (BSA, Sigma) with BSA standards (Smith *et al.*, 1985).

Citrate synthase activity

The activity of the citrate synthase was measured at the three experimental temperatures using the method of Thibault *et al.*, (1997). Samples were unfrozen and diluted to experimental concentration with HEPES-KOH (100 mM; pH=7,4). Essays were conducted in an imidazole-HCl (100mM, pH=8,0) in presence of 0,1 mM of DTNB, 0,1mM of actyl-coenzyme A and 0,15mM of oxaloacetic acid. Activity was monitored for three minutes and reaction rates were normalized to protein concentration of the homogenates as determined with bicinchoninic acid reagent (BSA, Sigma) with BSA standards (Smith *et al.*, 1985). Citrate synthase (CS) is a good marker of mitochondrial volume in tissues and we used this activity measured at 20°C as an indicator of mitochondrial content to normalize COX activity.

COX thermal stability

The thermal stability on the COX was measured on three extractions per species with CYC from horse heart. The homogenates were diluted with HEPES-KOH buffer (100 mM, pH=7,4) and incubated at 30°C for 36 hours. The activity of COX was measured polarographically at 0, 1, 2, 4, 8, 12, 24 and 36 hours of incubation. The assays were conducted in the same conditions used to determine the Km and Vmax and CYC at a concentration of 5 times the determined Km in polarography. Reaction rates were normalized to protein concentration of the homogenates as determined with bicinchoninic acid reagent (BSA, Sigma) with BSA standards (Smith *et al.*, 1985). The activity was first measured on freshly unfrozen samples and the activity obtained was considered as a reference. All subsequent activities were divided by this one to obtain the percentage of initial activity.

Comparison of cytochrome c concentration and its effect on the maximal velocity

To compare the maximal velocities obtained with those from other studies, we calculated velocities at published CYC concentration, using the Km and Vmax from the present study. The concentrations of CYC chosen for the calculation are commonly used in the literature when COX activity is measured using a spectrophotometer (50 and 100μM). Generated activities were then divided by our Vmax values to give a magnitude of error associated with the low concentrations of substrate used in many previous studies.

Statistical analysis

Km and Vmax were calculated using the enzyme kinetic module of Sigma-Plot version 7,101 (SPSS corp) using the Michaelis-Menten equations and statistical analysis were done with SYSTAT 9,0 (SPSS corp). Pair-wise comparisons were performed using LSD function.

Genetic analysis

Genetic analysis were performed on the proteins coding for the COX1 sub-unit All sequences were found in NCBI genbank (genbank accession number: honeybees AAM76450, arctic charr AAD41387, rabbits NP_659327, *N. virens* AAG32654, horse NP_007162, yeast NP_009305). Alignment of sequence data was carried using BioEdit 5.0.9 program. Phylogenetic analyses were conducted using PAUP phylogenetic software package 4,0. An equal weighted parsimony analysis was performed with yeast as the

outgroup. ACCTRAN character state optimization was used and bootstrap support for each clade was assessed with 1000 bootstrap replicates.

4. Results

Although not always significant, the impact of the origin of CYC is easy to detect, especially when combined with genetic analysis. Three-way analysis of variance show that Species (COX), CYC, temperature and all possible crosses between the three have significant effects on the affinity of COX for CYC. The same can be observed for the relative Vmax (ratio COX/CS_{20}, when CS is measured at 20°C), except for the CYC when considered alone.

Table 1. Comparison of kinetic properties of COX at different temperatures.

Effect	Df	SS	MS	F	P
Km					
COX	3	20,396	6,799	143,561	0,000
CYC	1	0,893	0,899	18,857	0,000
Temperature	2	3,072	1,536	32,434	0,000
COX x CYC	3	1,411	0,470	9.935	0,000
COX x Temp	6	4,312	0,719	15,176	0,000
CYC x Temp	2	5,812	2,911	61,461	0,000
COX x CYC x temp	6	4,373	0,729	15,390	0,000
Error	59	2,794	0,047		
COX/CS_{20}					
COX	3	1,952	0,651	9.912	0,000
CYC	1	0,198	0,198	3,024	0,087
Temperature	2	7,360	3,684	56,126	0,000
COX x CYC	3	2,332	0,777	11,840	0,000
COX x Temp	6	1,674	0,279	4,250	0,001
CYC x Temp	2	3,087	1,544	23,514	0,000
COX x CYC x temp	6	7,529	1,255	19,115	0,000
Error	68	4,464	0,066		

SS= Sum of square, MS = Mean square

Km and maximal velocity variations in relation to the source of CYC

At low (10°C) and medium (20°C) temperatures, warm-adapted species show a much lower Km value for yeast CYC than horse heart CYC whereas the opposite is found at 30°C. Differences have also been found for arctic charr but were detected at only one temperature (10°C). No difference has been detected for *N. virens*.

Three differences were detected when comparing the maximal velocities (figure 2a). Honeybees and rabbits show a much higher velocity at 30°C when in contact with CYC from yeast than CYC from horse. The opposite is found for *N.* virens, which shows a higher maximal velocity with horse heart CYC. No difference has been detected for arctic charr.

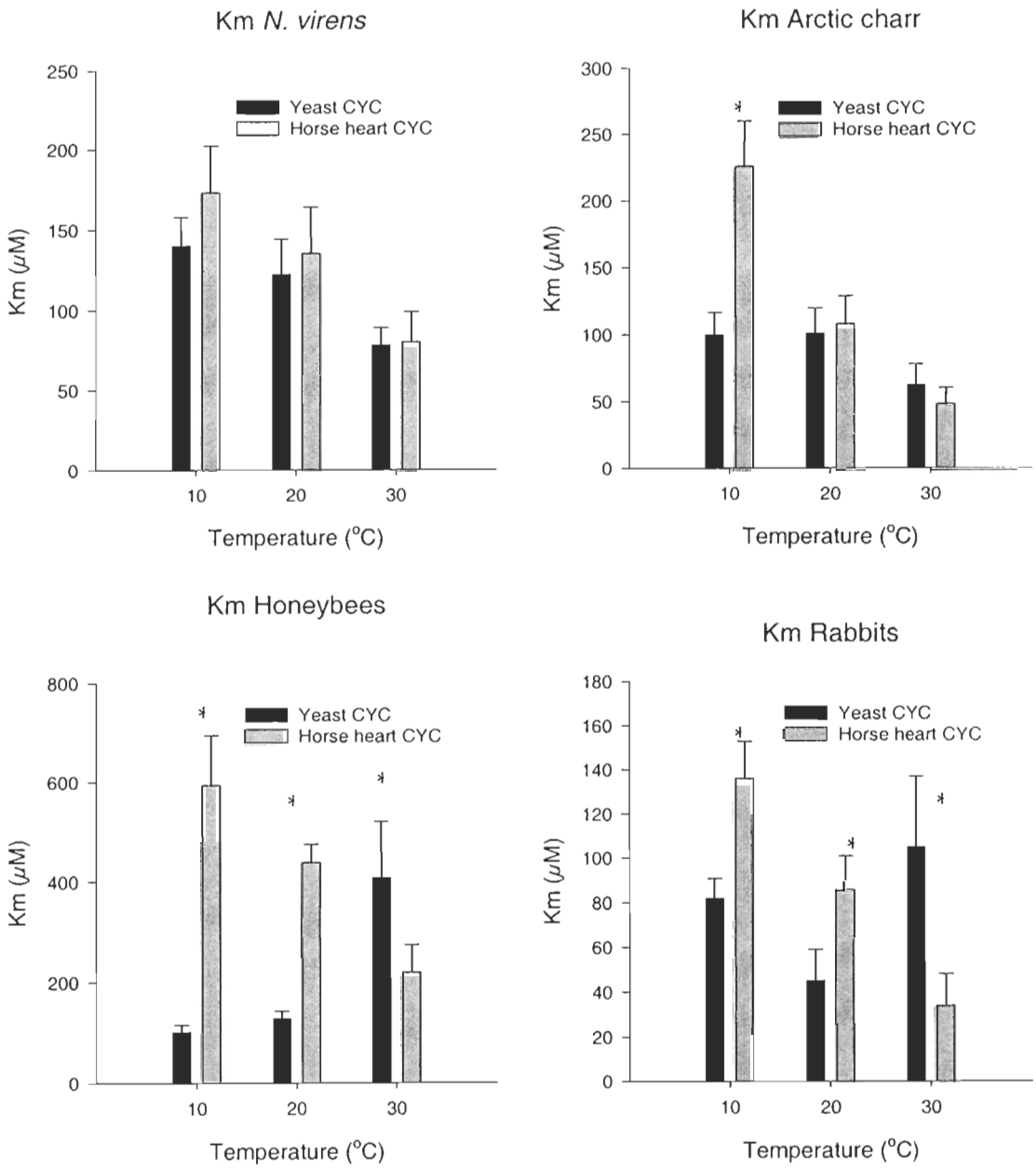
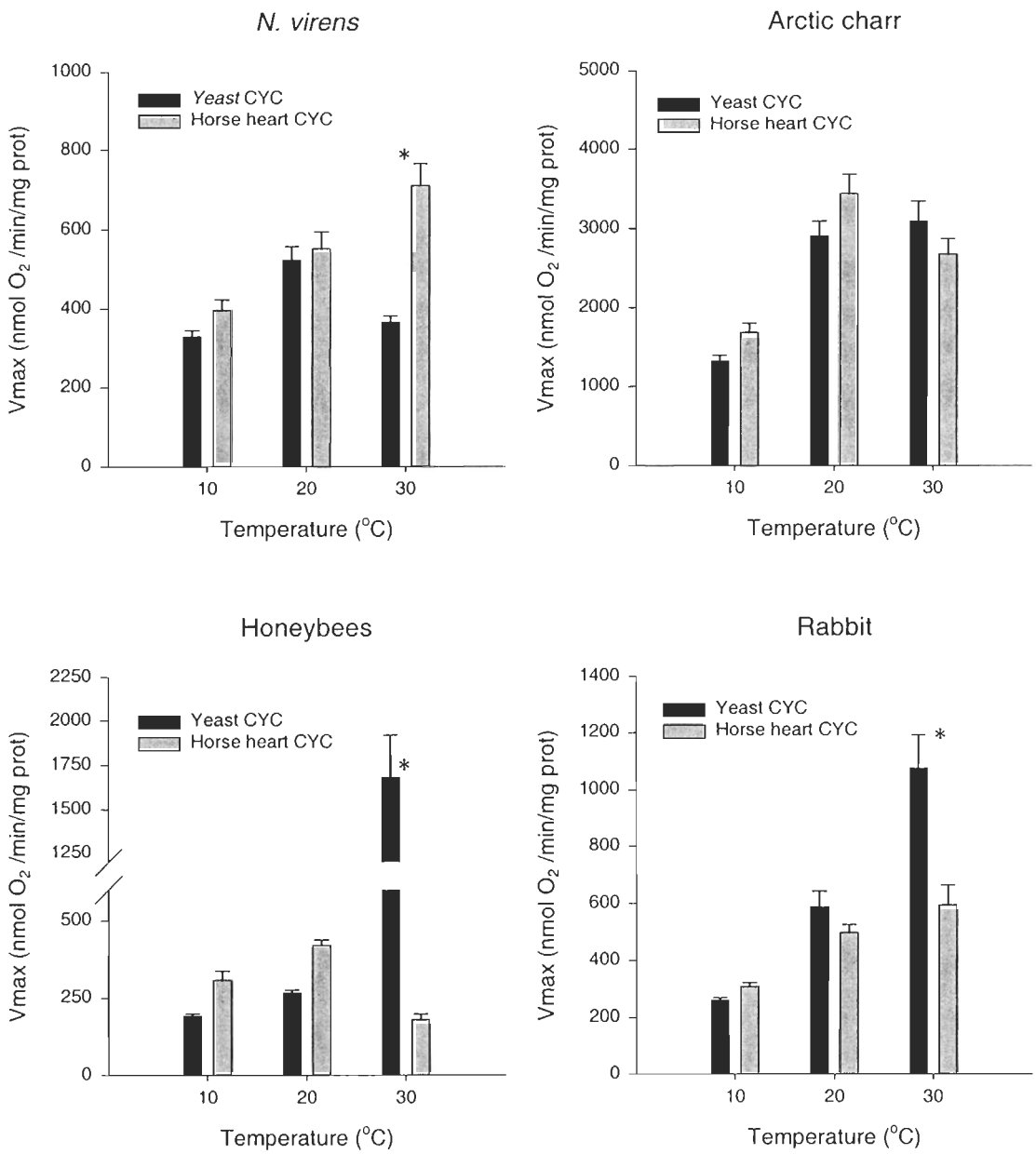


Figure 1. Km values of COX at three temperatures in relation with the origin of CYC.

(* $p < 0,05$; differences between the two CYC)

Arctic charr showed higher Vmax values, significantly different from all three other species at all temperatures (figure 2a). This could be attributed to a higher content in mitochondria in the red muscle and thus, higher content in COX in the extractions. In order to relativize its activity, COX Vmax is expressed in relation to another mitochondrial enzyme, the citrate synthase (CS) that is a good marker of mitochondrial content (figure 2b). This allows us to confirm that observed differences were caused by higher content of mitochondria. Such differences are less important for COX/CS and values of arctic charr are similar to those of other species.

A)


N. virens
Yeast CYC
Horse heart CYC
Vmax (nmol O2 /min/mg prot)
Temperature (°C)
Arctic charr
Yeast CYC
Horse heart CYC
Vmax (nmol O2 /min/mg prot)
Temperature (°C)
Honeybees
Yeast CYC
Horse heart CYC
Vmax (nmol O2 /min/mg prot)
Temperature (°C)
Rabbit
Yeast CYC
Horse heart CYC
Vmax (nmol O2 /min/mg prot)
Temperature (°C)

B)

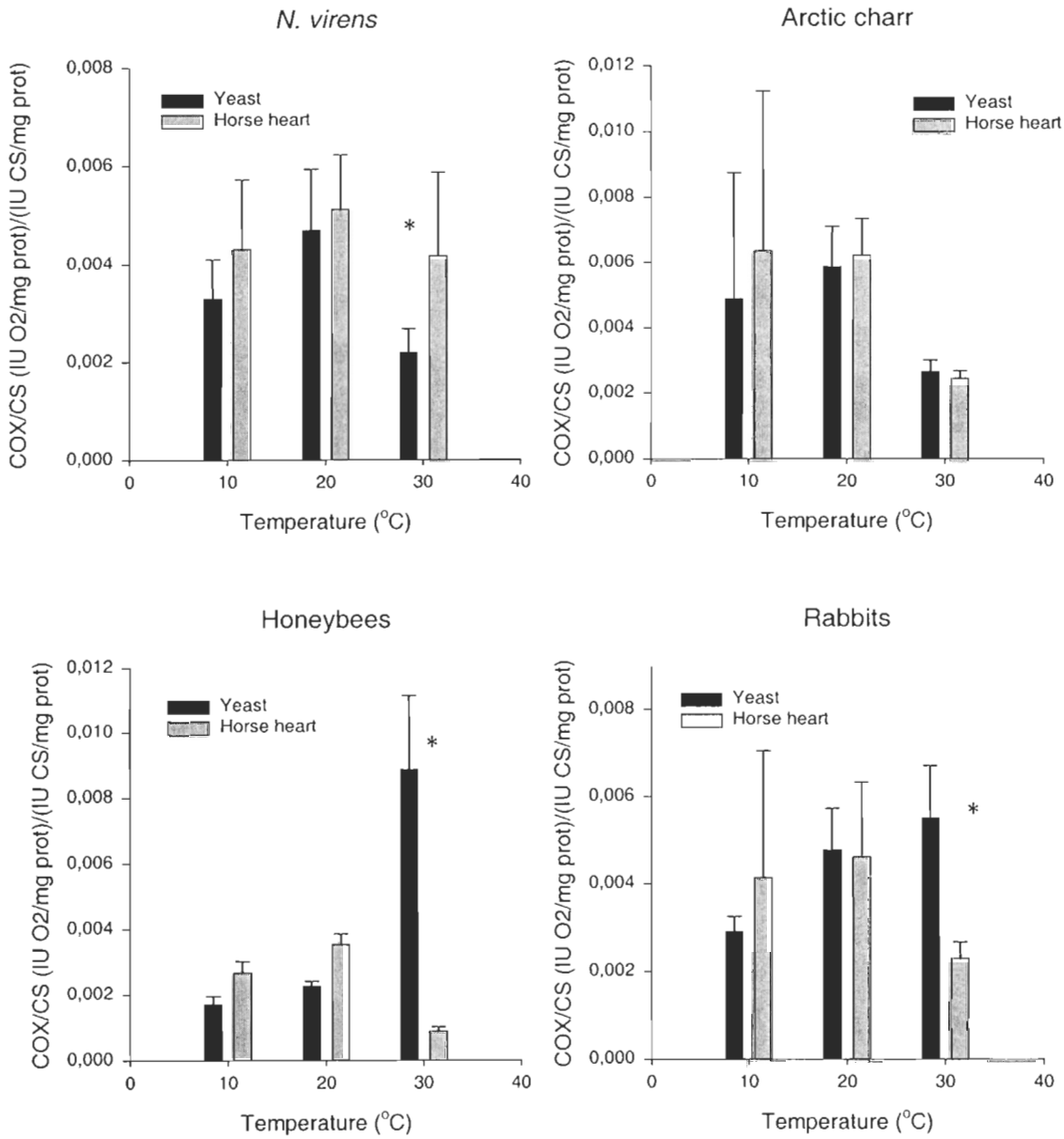


Figure 2. COX/CS activity in mitochondrial preparations from tissue of four different animals at three experimental temperatures.A) Activity expressed per mg of mitochondrial proteins B) activity expressed in relation with the activity of CS measured at each temperature (LSD pair-wise comparaison * : $p < 0,05$).

In order to remove the impact of the thermal sensitivity of citrate synthase and to highlight the impact of temperature on COX, we expressed COX activity at all three temperatures in function of the activity of CS measured at 20°C (figure 3). CS is a marker of mitochondrial content in our extractions and allows us to remove the influence of the quality of preparation in content of enzyme versus the total proteins purified. Significant differences observed in figure 2 are still present and at 10 and 20°C, honeybees show higher velocity when in contact with horse heart CYC. At low and medium temperatures, honeybees show lower velocities than other species when in yeast CYC. This impairment is inversed at low temperature.

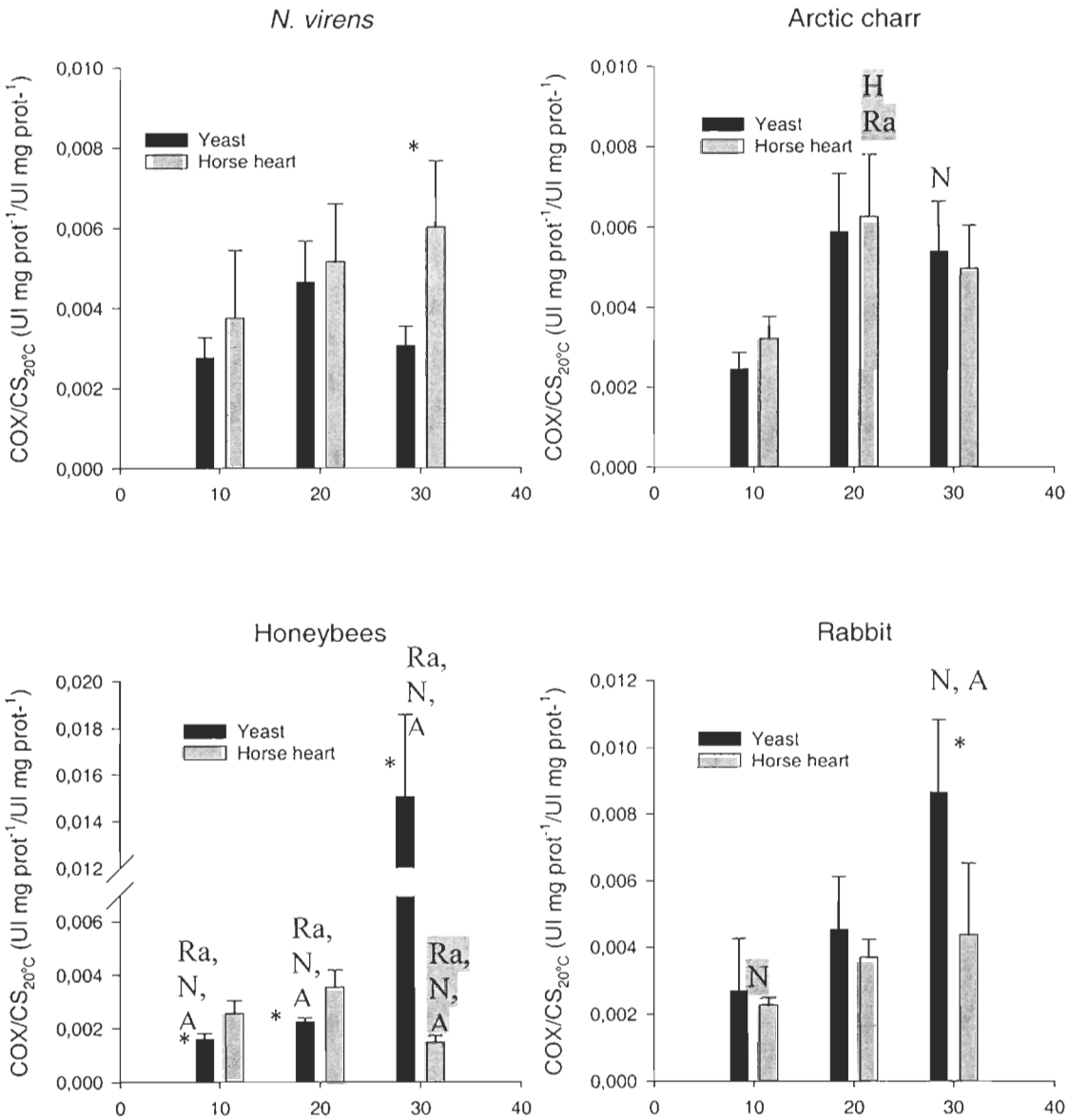


Figure 3. $COX/CS_{20°C}$ activity in mitochondrial preparations from tissue of four different animals at three experimental temperatures when expressed in relation with the origin of the CYC (letter indicate a significant difference between the species and : rabbit (Ra), arctic charr (A), N. virens (N) or honeybees (H). Shadowed letter are for horse heart whereas unshadowed are for yeast CYC. (*LSD pair-wise comparaison : $p < 0,05$).

Impact of temperature on the Km and Vmax of COX

Although not always significant, a general tendency toward a decrease of the Km value with the increase of the reactive temperature can be observed when CYC from horse is present but not for the cytochrome c from yeast (figure 1). Only two species, arctic charr and *N. virens*, show such a pattern when in contact with yeast's CYC; the two others show a reverse trend between 20 and 30°C. Warm-adapted species show a clear tendency (decrease of Km) when in contact with CYC from horse heart and cold-adapted species show the same when in contact with either horse heart or yeast CYC.

Warm-adapted species show a significant increase of the maximal velocity with temperature increasing from 10°C to 30°C when CYC from yeast is used. Only small differences between the relative maximal velocities reached were detected with CYC from horse heart (figure 2a). When this CYC is used, rabbits show a tendency for an increase of the maximal velocity that is not found for honeybees. Arctic charr show the same pattern for both sources of CYC; an increase of the Vmax between 10°C and 20-30°C. A tendency toward an increase in Vmax at higher temperature is found for *N. virens*, but only one significant difference was detected.

Interspecific variation of the Km and maximal velocity

Several differences were detected between the Vmax of species. Arctic charr showed much higher Vmax than the three other species at all temperatures. Even though the same experimental protocol was used and as lateral red muscle from fish contains high concentrations of mitochondria, a high concentration of COX might have been present in the extractions from arctic charr. In consequence, inter-specific difference will be considered only when expressed in relation with a mitochondrial content marker (CS). This allowed to minimize this impact of the quality of enzymatic preparation and showed that COX activities of arctic charr are similar to those obtained from other species.

Q10 values

A three-way analysis of variance show that Species (COX), CYC, temperature and all possible crosses between the three have significant effects on the values of Q_{10} of COX activity. Only one significant difference among species was detected when comparing the Q_{10} obtained between 10 and 20°C; honeybees show a lower value than arctic charr when horse heart CYC is used. Honeybees show significant differences with all other species for Q_{10} between 20 and 30°C and 10 to 30°C (figure 4) with both CYC. Depending of the CYC used, two patterns are observed. With horse heart CYC, honeybees show a lower Q_{10} than all other species between 20 and 30°C and 10 to 30°C whereas the opposite is found for CYC from yeast. Indeed, when yeast CYC is used, honeybees show a higher thermal sensitivity than all other species at the two temperature ranges. Arctic charr also show significant differences with N. virens and rabbits between 10 and 30°C.

Table 2. Comparison of the thermal sensitivity of COX.

Effect	Df	SS	MS	F	P
COX	3	0,907	0,302	7,027	0,000
CYC	1	3,316	7,316	77,035	0,000
Temperature	2	2,397	1,199	27,843	0,000
COX x CYC	3	8,255	2,752	63,919	0,000
COX x temperature	6	2,728	0,455	10,56	0,000
CYC x temperature	2	1,276	0,638	14,827	0,000
COX x CYC x temperature	6	6,151	1,025	23,814	0,000
Error	56	2,411	0,043		

SS= Sum of square, MS = Mean square

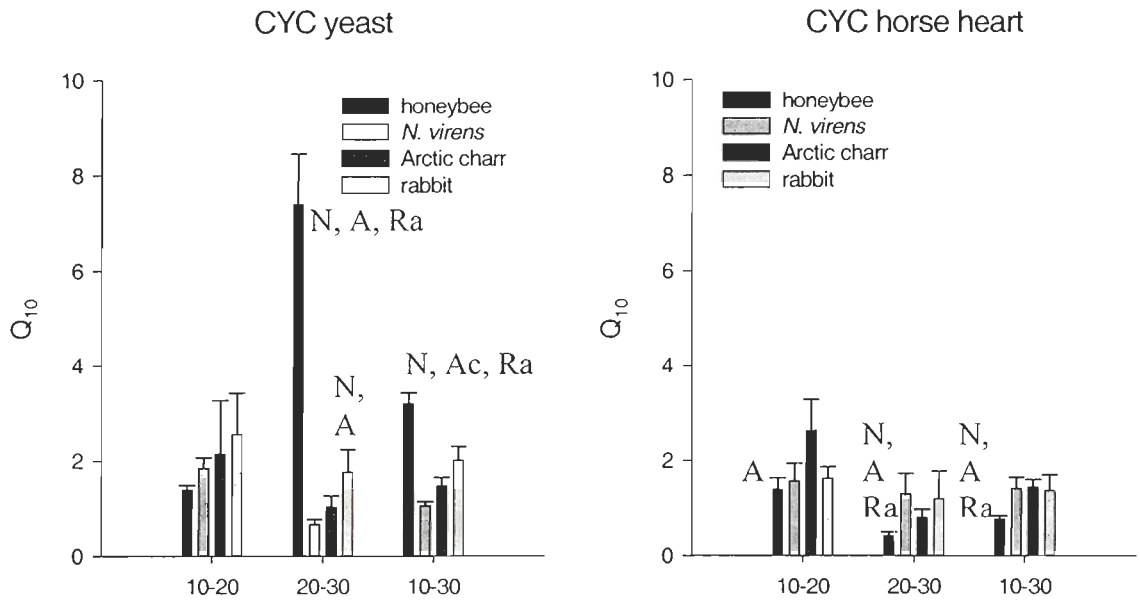


Figure 4. Inter-specific Q10 comparaison for all species in relation with the type of CYC used. Letter indicate a significant difference between the species (rabbit (Ra), arctic charr (A), N. virens (N) or honeybees (H)).

When comparing the impact of temperature range on Q_{10} once again honeybees show different patterns depending of the CYC present. With yeast, the Q_{10} (20-30°C) is higher than the Q_{10} (10-20)°C whereas with horse heart CYC it shows a smaller Q_{10} value. Arctic charr also show significant differences but the same pattern is observed for the two CYC: Q_{10} (10-20°C) is higher than Q_{10} (20-30°C).

Thermal stability of COX

The four species tested for thermal stability show very clear patterns. Rabbits maintained their initial activity after 24 hours of incubation at 30°C. Honeybees lost activity much faster; almost 50% within the first hour. Cold-adapted species were able to maintain activity longer than honeybees, but still lost activity faster than rabbits; almost 60% of the activity was lost within the 4 first hours for *N. virens* and 25% for arctic charr for the same period. After two hour of incubation, rabbits showed a significantly higher relative activity than other species and that difference is found in all subsequent essays (figure 5). These results suggest a high thermal stability of the rabbit enzyme compare to the other species, although we expected a good stability at 30°C for honeybees.

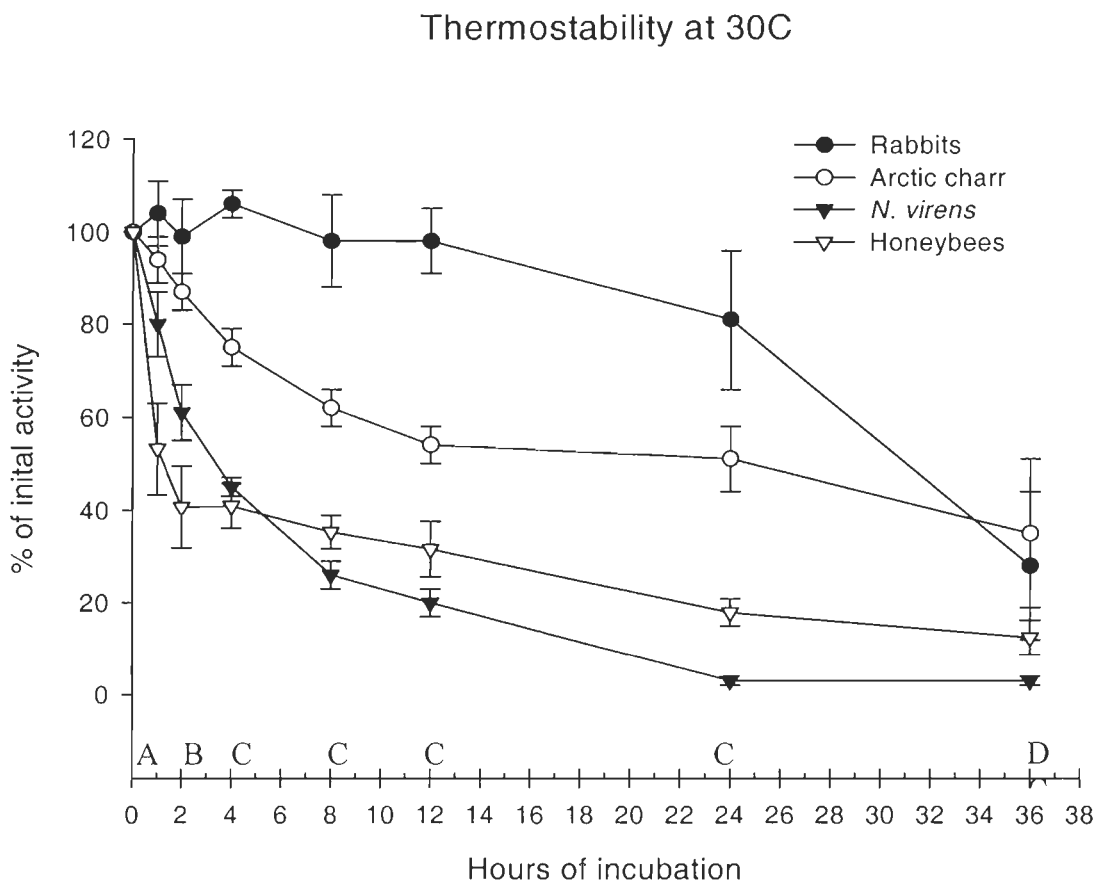


Figure 5. Thermal stability of COX when incubated at 30°C. Activities were divided by the initial activity to give a percentage of the initial activity (letter express significant differences between species LSD test).

A: at 1 hour of incubation: N. virens > Honeybees; Arctic charr and rabbits > *N. virens*

B: at 2 hours of incubation rabbits > arctic charr > *N. virens* > honeybees

C: at 4, 8, 12 and 24 hours of incubation: rabbits > arctic charr > honeybees > *N. virens* (at 4 hours : honeybees = N. virens)

D: at 36 hours of incubation : rabbits and arctic charr > *N. virens*

Genetic analysis

Genetic analyses show that yeast is a sister-group to all other species and that horse and rabbit are closely similar. Honeybees are the test-species that is genetically furthest from horse.

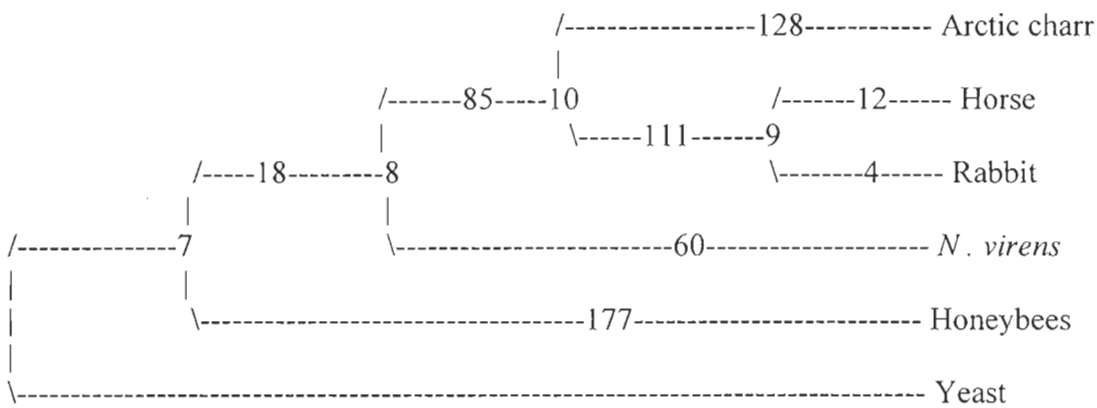


Figure 6. Genetic analysis of the sub-unit COX1. Alignment of sequence data was carried using BioEdit 5.0.9 program. Phylogenetic analyses were conducted using PAUP phylogenetic software package 4,0. An equal weighted parsimony analysis was performed with *S. cerevisae* as outgroup. ACCTRAN character state optimization was used and bootstrap support for each clade was assessed with 1000 bootstrap replicates. Numbers represent the amount of steps between the node and the species.

Comparison of CYC concentration and its effect on the maximal velocity of COX

The velocity values generated form our kinetic slope with substrate concentration used in the literature are systematically lower than the Vmax found in the present study. These values under-estimate the Vmax and represent between 7,75 and 74,18% of our Vmax, depending on the species and CYC concentration (figure 6). No statistical analyses were performed on the two set of data.

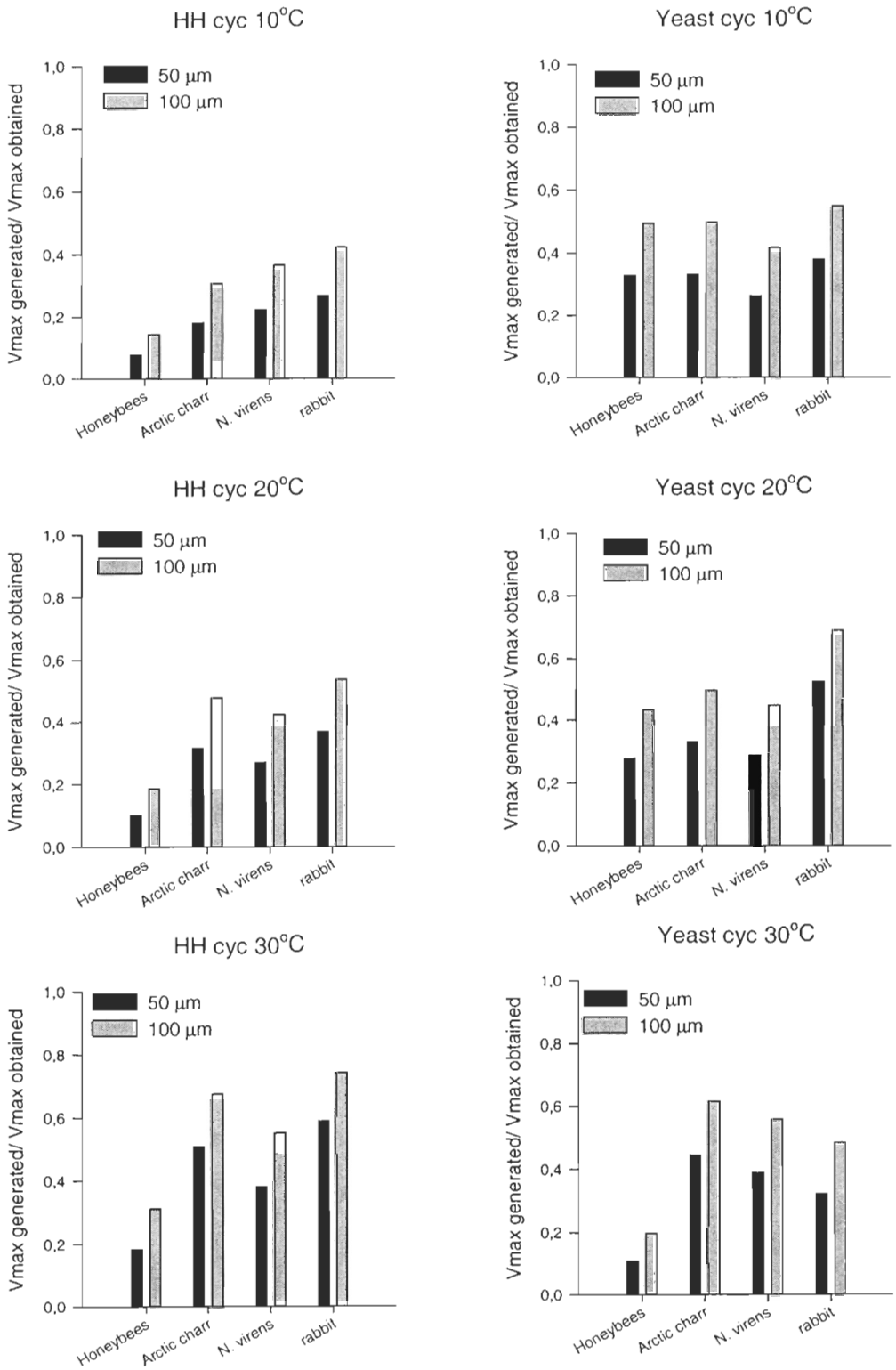


Figure 7. Activities of COX at two concentrations of CYC frequently used in the. litterature. Activities have been estimated from our kinetics values.

5. Discussion

The four species studied are very distinct phylogenetically, indicating long independant evolutionary histories. The genetic analysis showed all species to be more closely-related to each other than to yeast. Considering the phylogenetic tree, if genetic distance between the species is the main factor influencing the functional properties, we could expect to find similar responses when using yeast's CYC and different responses with horse's CYC, depending of the genetic distance between horse and the test-species. The species can be separated in two classes: warm-adapted (rabbits and honeybees) and cold-adapted (*N. virens* and arctic charr) species. One of our first initial assumptions was that the results would outline two groups: warm-adapted and cold-adapted species. It turned out that although similar results were found within each group, they would have to be broken apart to allow a better analysis. Our results show that temperature has a significant impact on the affinity and maximal velocities on the COX with all species showing a decrease of Km as temperature increases when CYC form horse heart is used. Only warm-adapted species (rabbit and honeybees) show important variations of their Km values when CYC from yeast is used.

Thermal stability at high temperature

The fast denaturation of enzyme at high temperature has long been recognized (Zecchinon *et al.*, 2001; Feller, 2003). The rate of inactivation depends mainly of the natural thermal environment of the enzyme, ranging from few minutes at 50-60°C for psychrophilic

enzymes to few hours for warm-adapted species (Feller, 2003). Considering previous studies on warm-adapted organisms and the mechanism of thermal adaptations, we expected a good stability at 30°C for both warm-adapted species, but it turned out that honeybees COX was among the fastest to be inactivated. Fast-inactivation of cold-adapted enzymes have been reported in many studies (Zecchinon *et al.*, 2001), but such results for honeybees seemed quite difficult to explain. Activity of enzymes included in membranes is influenced by the phospholipids surrounding it and the fluidity they give to the membrane. An increase in the proportions of ethanolamine phospholipids in the membrane of cold-adapted species has oftenly been observed (Wodtke, 1981). When exposed to high temperature, such membranes tend to be instable leading to fast inactivation of their enzymes. The inactivation rate found for arctic charr and *N. virens* could support such assumptions. Another important factor of the flexibility is the content of cholesterol within the membrane (Crockett and Hazel, 1995). Cholesterol increases the rigidity thus leading to a decrease of flexibility and impairs the ion leak of the membrane. Mammals are known to present higher content of cholesterol within their membranes than fish, therefore being more stable at high temperature. As honeybees need high body temperature to maintain their catalytic properties (Moffatt, 2001), we could expect a high content in cholesterol in order to rigidify their membranes, and thus avoid instability and proton-leaks . However, Suarez *et al.*, (2000) have shown that their metabolim is very high (307,7 umol O_2 g^{-1} s^{-1}), which could be associated with very fluid membranes. Moreover, Gimpl *et al.*, (1995) reported a very low content of cholesterol in the membrane of insects; reaching 10 times less that mammal concentrations. This low content of cholesterol could imply a high flexibility of the membrane, allowing high enzymatic activity to perform stationary flight,

but also very little stability leading to the high thermal denaturation found in the present study. Further studies should look more deeply at the impact of membrane composition on the functional properties and thermal stabilities of bees mitochondria in regard to the high energetic requirement of flight. Our results suggest a "smooth" structure of COX that preclude a high catalytic capacity of mitochondria which could be interpreted as an adaptation to high energetic performance at the expense of temperature stability.

Impact of temperature on COX

Our results show a simultaneous increase of the V_{max} and decrease of Km with temperature when horse heart cytochrome c is used. This tendency is quite challenging since previous studies showed an opposite trend of these functional properties when studying other enzymes (for review see Feller (2003)). However, most enzymes studied are water-soluble, for example LDH (Holland *et al.*, 1997) and α-amylase (D'Amico *et al.*, 2003), presenting mainly weak-interactions. The strength of such bonds decreases with an increase of the temperature. However, COX is a membrane-bound enzyme and such enzymes have been reported to be more stable than water-soluble enzyme (Hardewig *et al.*, 2000) through dependence upon hydrophobic bonds (O'brien *et al.*, 1991). Whereas other weak-bond losses strength, hydrophobic bonds show the opposite tendency, their strength increases with temperature. As these links have important roles to play in the stability of the folded protein conformation and thus, increasing their strength implies to stabilize the conformation of the enzyme bearing the active site. That could facilitate the binding of the substrate to the active site, lowering the Km.

Johnston *et al.*, (1994) found a little degree of temperature compensation of the maximal mitochondrial respiration rates when studying the thermal tolerance of mitochondria from warm-adapted species. If compensation had taken place, all Km or Vmax from all species would tend to a similar value at their environmental or optimal temperature. In our study, the closest pair of COX and CYC is rabbit and horse, both thermally and phylogenetically (figure 6). Therefore we can consider the kinetic properties of rabbit COX when using horse CYC as a ''reference''. At physiological temperature, the Km (35 uM) is consistent with previously reported Km for other endotherms: *Bos taurus* muscle (20 μM Sinjorgo *et al.*, 1987; Sinjorgo *et al.*, 1988) and *Rattus norvegicus* (30 μM; Lee and Lee, 1995; Schagger *et al.*, 1995). At their physiological temperatures, all three others species, show Km values around 200 μM when measured with horse heart CYC. However, when closer to physiological temperature, the use of yeast CYC with cold-adapted species lowers the Km values, and thus brings them closer to the reference. Moreover, little or no compensation was found for the maximal velocities either. The general trend is an increase of activity from 10°C to 20°C while between 20°C and 30°C the activities decreased for arctic charr and bees COX when horse CYC is used as substrate and for *N. virens* COX when yeast CYC is used. However, maximal velocity is around 250 U/mg protein at 10°C for honeybees and rabbit while it goes from 350 (*N. virens*) to approximately 1250 U/mg protein (arctic charr). Such results can suggest a partial temperature compensation of maximal speed of the enzyme and a conservation of the affinity. This also suggest that in addition to catalytic capacity, Km value (or affinity) should be an important criteria to consider when studying adaptation of COX at different temperatures or studying

coadaptation of nuclear and mitochondrial genome. Furthermore, the fact that honeybees show the same tendencies as rabbits, suggests that temperature of adaptation is a good predictor of the properties of COX.

All species show approximately the same tendency for Km and Vmax when CYC from horse heart is used. Their Km value decreases with temperature as their Vmax increases. Moreover, Blier and Lemieux, (2001) showed in arctic charr muscle preparations that over 90% of COX had to be inhibited by potassium cyanide (KCN) before any change in the respiration rate of mitochondria could be detected. This result suggest that the enzyme is in excess and rather than the enzyme catalytic capacity, the substrate availability could be a limiting condition *in vivo*. Therefore, affinity of the enzyme for its substrate would be a key parameter to protect in a space-limited environment where substrate availability is restricted, such as inner membrane of mitochondria. Our results show that Km is more protected than Vmax and combined with hypothesis expressed by Suarez *et al.*, (1996), Edmands and Burton, (1998), Suarez *et al.*, (2000) and Blier and Lemieux, (2001), it suggests an important impact of COX on the maintenance and regulation of a proper level of redox potential compatible with electron fluxes needed to maintain the regulation mechanisms throughout the ETC.

Comparison of methods of COX activity measurements

Being an important step of the mitochondrial metabolism (Blier and Lemieux, 2001) and an important factor of the oxidative phosphorylation, COX activity has been studied in many previous studies. In numerous studies its activity has been used as a marker of mitochondrial content and aerobic capacity. Two main methods have been used to quantify the activity of this enzyme, either spectrophotometrically (Edmands and Burton, 1998; Blier and Lemieux, 2001) or polarographically (O'brien *et al.*, 1991). The first is based on the spectral absorption of reduced cytochrome c at 550nm, whereas the second monitors the consumption of O_2 during the reduction of CYC by COX. Many studies used spectrophotometry mainly because of the low reactive volume and the ease of work allowed by the technique. However this method has a major drawback: the limit in absorbance of the spectrophotometer. Because of the high extinction coefficient of ferrocytochrome c (= 29,1 mM^{-1} cm^{-1}) the sensor is easily saturated at relatively low concentration of substrate. Wiedemann *et al.*, (2000) reported that at concentrations higher than 80 uM of CYC, the technique was not applicable anymore. As general rule, many authors suggest to measure the Vmax at 10 times the Km of the enzyme, forcing us to work with high concentrations of cytochrome c well-above the limit of detection of spectrophotometer. Many previous studies, using COX as a marker of mitochondrial activity, have used much lower concentrations, usually from 50 to 100 μM of cytochrome c (Suarez *et al.*, 1996; Edmands and Burton, 1998; Blier and Lemieux, 2001). These concentrations are lower than Km values found in the present study, and thus do not allow a proper evaluation of the maximal velocity reached by the enzymes. The velocities obtained

in the present study at these concentrations represent between 7,75 to 74,18 % of the Vmax depending on temperature and cytochrome c origin, which represent an important under-estimation of the activity of the enzyme. Honeybee is the species showing the highest differences considering methods and concentrations. As this species shows very high Km values, the use of low concentrations of substrate implies an important under-estimation on the maximal velocity reached by the enzyme.

6. Conclusion

The investigation of co-evolution of an enzyme encoded by the mitochondria and a protein encoded by the nucleus can be a perfect opportunity to study the impact of temperature as a selective pressure. The results of the present study suggest that co-evolution has taken place, and that temperature could have been a pressure of positive selection, as well as a significant evolutionary drive allowing species to adapt to several environments.

The genetic analysis of the COX1 sub-unit from the six studied species showed a "classical" phylogenetic tree. However, physiological analysis has proven that species from similar thermal environment show the same trends, thus suggesting that the temperature of adaptation, when compared to phylogenetic distance, is a good predictor of functional properties.

We also showed that the affinity of COX toward its substrate may be influenced by the origin of CYC. When COX properties of distant species but adapted to similar thermal

environment are studied, the origin of the substrate (CYC) could explain important variations. Therefore, the use of CYC from a distant species can cause important impairment of the maximal velocities obtained by studies on functional properties and should be taken in account in further studies.

Finally, we demonstrated that measurement of COX activity using spectrophotometry systematically under-estimate the Vmax values. This technique is limited by the use of low concentrations of substrate, giving velocities clearly lower than velocities obtained using polarographyat higher concentrations.

7. Acknowledgements

The authors would like to thank Armelle Barroux and Caroline Asselin for their assistance in developing and testing experimental protocols used. This research was supported by funds provided by NSERC to P.U. Blier. B. Auclair received the Sébastien Boisvert post-graduate scholarship awarded by the FUQAR.

Chapitre 3. Conclusion

Nos objectifs au cours de la présente étude, étaient de déterminer l'impact de la température sur les propriétés fonctionnelles d'une enzyme-clé du métabolisme mitochondrial. Contrairement aux concepts attendus des théories actuelles de l'évolution, nos résultats suggèrent fortement que cette enzyme a été soumise à une forme de sélection positive favorisant des adaptations permettant le maintien des propriétés fonctionnelles et de la régulation des mécanismes aux différentes températures auxquelles chaque organisme est acclimaté. Ainsi, il semble que la température ait été un moteur significatif de l'évolution, permettant aux organismes de s'adapter à divers milieux.

Nous pouvons émettre l'hypothèse que plusieurs mutations présentes dans les gènes de la COX ne sont plus simplement fixées aléatoirement dans les génomes, mais peuvent être positivement sélectionnées. Ceci ajoute aux réserves de plus en plus nombreuses concernant les études utilisant strictement des outils génétiques, car celles-ci sont limitées dans leurs analyses par les méthodes utilisées qui ne permettent pas de distinguer les mutations importantes. Les analyses physiologiques permettent de quantifier et relativiser les conséquences des diverses mutations sur le phénotype des espèces.

Les conclusions obtenues ne peuvent, par contre, être uniquement expliquées par des mutations. Nos résultats suggèrent également un rôle important de la composition et donc, de la fluidité membranaire. Tel que démontré dans de nombreuses études, celle-ci influence de façon significative les propriétés fonctionnelles des enzymes. Ceci est particulièrement mis en évidence chez l'abeille qui présente des mitochondries démontrant

une organisation permettant de très hautes capacités catalytiques au détriment de la stabilité thermique, ce qui peut être corrélée à la composition membranaire. Ces mitochondries représentent donc un modèle parfait pour l'étude de l'impact la composition membranaire sur les propriétés fonctionnelles et la stabilité thermique en relation avec les nécessités du vol.

De plus, les tendances détectées lors de l'analyse des variations du Vmax et du Km en fonction la température, montrent clairement une compensation partielle de la vitesse maximale de l'enzyme et une conservation de l'affinité pour le substrat à différentes températures. Les vitesses maximales allant de 250 U/mg protéines à 10°C (pour l'abeille et le lapin) à 350 et approximativement 1250 U/mg protéines pour N. virens et l'omble chevalier respectivement, suggèrent que contrairement à ce qui a été fait antérieurement, en plus de valeurs de Vmax, les valeurs de Km (ou d'affinité) devraient être considérées comme critère important à considérer lors d'études sur la co-évolution de génome nucléaire et mitochondrial. Ceci confirme l'hypothèse avancée par Blier et Lemieux Blier and Lemieux, (2001) et montre que l'affinité est un facteur clé à maintenir dans un environnement où l'espace ainsi que la disponibilité du substrat peuvent être limitant et contraignants, par exemple à l'intérieur de la membrane interne des mitochondries.

Il semble ainsi possible que lors d'études portant sur le propriétés fonctionnelles de la COX, l'origine du substrat soit responsable d'une part importante des variations observées lorsque de grandes différences sont présentent entre les températures d'acclimatation des espèces étudiées. Par contre, à faible différence de température, la COX devrait être un

facteur important de la variation observée. En effet, nos résultats montrent que le choix de l'espèce d'origine du substrat peut influencer de façon importante les résultats obtenus dans des études physiologiques portant sur la COX. Des espèces phylogénétiquement éloignées mais s'étant adaptées à des environnements thermiques semblables possèdent des enzymes présentant des valeurs d'affinité enzymatiques très similaires. Ainsi, les conclusions seront grandement influencées, non pas par les conditions expérimentales, mais davantage par les adaptations propres à chaque environnement thermique. À l'inverse, l'origine du substrat n'aura que très peu d'impact lors d'études entre des espèces adaptées à des environnements thermiquement distincts. Par exemple, lors d'études portant sur les propriétés fonctionnelles de la COX utilisant comme espèces expérimentales l'omble chevalier, adaptée à des environnements froids, et l'omble de fontaine (*S. fontinalis*), adaptées à des milieux plus chaud, le choix du cytochrome c aura davantage d'impact car les espèces sont de milieux thermiques différents bien qu'elles soient de statut phylogénétique proche. Le rôle du substrat ne serait pas si important dans le cadre d'une étude comparative entre la COX d'abeille et de cheval, car bien que phylogénétiquement distantes, elles présentent des environnements thermiques d'adaptation similaires.

Finalement, il ressort de nos conclusions que l'utilisation de la spectrophotométrie pour mesurer la vitesse maximale de la COX implique systématiquement une sous-estimation de la valeur du Vmax. Cette technique, bien que facile d'utilisation, nécessite l'utilisation de faible quantité de substrats lors des dosages enzymatiques. Ces concentrations étant sous les valeurs de saturation enzymatique en substrat donne des valeurs de vitesse nettement

inférieures (entre 7,75 et 74,18% de la valeur obtenue par polarographie) aux valeurs de Vmax obtenues par polarographies.

Références bibilographiques

Allen, J. F. (1993). "Control of Gene-Expression by Redox Potential and the Requirement for Chloroplast and Mitochondrial Genomes." Journal of Theoretical Biology **165** (4): 609-631.

Allen, J. F. and J. A. Raven (1996). "Free-radical-induced mutation vs redox regulation: Costs and benefits of genes in organelles." Journal of Molecular Evolution **42** (5): 482-492.

Barrientos, A., S. Muller, R. Dey, J. Wienberg and C. T. Moraes (2000). "Cytochrome c oxidase assembly in primates is sensitive to small evolutionary variations in amino acid sequence." Molecular Biology and Evolution **17** (10): 1508-1519.

Berg, O. G. and C. G. Kurland (2000). "Why mitochondrial genes are most often found in nuclei." Molecular Biology and Evolution **17** (6): 951-961.

Blier, P. U., F. Dufresne and R. S. Burton (2001). "Natural selection and the evolution of mtDNA-encoded peptides: evidence for intergenomic co-adaptation." Trends in Genetics **17** (7): 400-406.

Blier, P. U. and H. Lemieux (2001). "The impact of the thermal sensitivity of cytochrome c oxidase on the respiration rate of Arctic charr red muscle mitochondria." Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology **171** (3): 247-253.

Burton, R. S., P. D. Rawson and S. Edmands (1999). "Genetic architecture of physiological phenotypes: Empirical evidence for coadapted gene complexes." American Zoologist **39** (2): 451-462.

Crockett, E. L. and J. R. Hazel (1995). "Cholesterol levels explain inverse compensation of membrane order in brush border but not homeoviscous adaptation in basolateral membranes from the intestinal epithelia of rainbow trout." Journal of Experimental Biology **198** (5): 1105-1113.

D'Amico, S., C. Gerday and G. Feller (2003). "Temperature adaptation of proteins: Engineering mesophilic-like activity and stability in a cold-adapted alpha-amylase." Journal of Molecular Biology **332** (5): 981-988.

Edmands, S. and R. S. Burton (1998). "Variation in cytochrome-c oxidase activity is not maternally inherited in the copepod Tigriopus californicus." Heredity [Heredity] **80** (6): 668-674.

Edmands, S. and R. S. Burton (1999). "Cytochrome C oxidase activity in interpopulation hybrids of a marine copepod: A test for nuclear-nuclear or nuclear-cytoplasmic coadaptation." Evolution **53** (6): 1972-1978.

Feller, G. (2003). "Molecular adaptations to cold in psychrophilic enzymes." Cellular and Molecular Life Sciences **60** (4): 648-662.

Gimpl, G., U. Klein, H. Reilander and F. Fahrenholz (1995). "Expression of the Human Oxytocin Receptor in Baculovirus-Infected Insect Cells - High-Affinity Binding Is Induced by a Cholesterol Cyclodextrin Complex." Biochemistry **34** (42): 13794-13801.

Gray, M. W., G. Burger and B. F. Lang (1999). "Mitochondrial evolution." Science **283** (5407): 1476-1481.

Hardewig, I., P. L. M. van Dijk, S. C. Leary and C. D. Moyes (2000). "Temporal changes in enzyme activity and mRNA levels during thermal challenge in white sucker." Journal of Fish Biology **56** (1): 196-207.

Hazel, J. R. (1995). "Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation?" Annual review of physiology **57**: 19-42.

Hochachka, P. W. and G. N. Somero (2002). Biochemical adaptations; Mechanism and process in physiological evolution. New York, Oxford University Press.

Holland, L. Z., M. McFall-Ngai and G. N. Somero (1997). "Evolution of lactate dehydrogenase-A homologs of barracuda fishes (genus Sphyraena) from different thermal environments: Differences in kinetic properties and thermal stability are due to amino acid substitutions outside the active site." Biochemistry (Washington) [Biochemistry (Wash.)] **36** (11): 3207-3215.

Hutter, C. M. and D. M. Rand (1995). "Competition between Mitochondrial Haplotypes in Distinct Nuclear Genetic Environments - Drosophila-Pseudoobscura Vs D-Persimilis." Genetics **140** (2): 537-548.

Johnston, I. A., H. Guderley, C. E. Franklin, T. Crockford and C. Kamunde (1994). "Are Mitochondria Subject to Evolutionary Temperature Adaptation." Journal of Experimental Biology **195**: 293-306.

Kenyon, L. and C. T. Moraes (1997). "Expanding the functional human mitochondrial DNA database by the establishment of primate xenomitochondrial cybrids." Proceeding of the National Academy of Science USA **94**: 9131-0135.

Kimura, M. (1992). La théorie neutraliste de l'évolution. Paris, Flammarion.

Kurland, C. G. and S. G. E. Andersson (2000). "Origin and evolution of the mitochondrial proteome." Microbiology and Molecular Biology Reviews **64** (4): 786-+.

Lang, B. F., M. W. Gray and G. Burger (1999a). "Mitochondrial genome evolution and the origin of eukaryotes." Annual review of genetics **33**: 351-397.

Lang, B. F., E. Seif, M. W. Gray, C. J. O'Kelly and G. Burger (1999b). "Comparative genomics approach to the evolution of eukaryotes and their mitochondria." Journal of Eukaryotic Microbiology **46** (4): 320-326.

Lee, J. Y. and S. J. Lee (1995). "Enzymatic-Properties of Cytochrome-Oxidase from Bovine Heart and Rat-Tissues." Journal of Biochemistry and Molecular Biology **28** (3): 254-260.

Lehninger, A. L., D. L. Nelson and M. M. Cox (1994). Principes de biochimie. Paris, Flammarion.

Moffatt, L. (2001). "Metabolic rate and thermal stability during honeybee foraging at different reward rates." Journal of Experimental Biology **204** (4): 759-766.

O'brien, J., E. Dahlhoff and G. N. Somero (1991). "Thermal resistance of mitochondrial respiration: hydrophobic interactions of membrane proteins may limit thermal resistance." Physiological zoology **64** (6): 1509-1526.

Rand, D. M., A. G. Clark and L. M. Kann (2001). "Sexually antagonistic cytonuclear fitness interactions in Drosophila melanogaster." Genetics **159** (1): 173-187.

Rawson, P. D. and R. S. Burton (2002). "Functional coadaptation between cytochrome c and cytochrome c oxidase within allopatric populations of a marine copepod." Proceedings of the National Academy of Sciences of the United States of America **99** (20): 12955-12958.

Reynafarje, B., L. E. Costa and A. L. Lehninger (1985). "0xygen solubility in aqueous media determined by a kinetic method." Analytical biochemistry **145**: 406-418.

Sackton, T. B., R. A. Haney and D. M. Rand (2003). "Cytonuclear coadaptation in Drosophila: Disruption of cytochrome c oxidase activity in backcross genotypes." Evolution **57** (10): 2315-2325.

Schagger, H., H. Noack, W. Halangk, U. Brandt and G. Vonjagow (1995). "Cytochrome-C-Oxidase in Developing Rat-Heart - Enzymatic-Properties and Amino-Terminal Sequences Suggest Identity of the Fetal Heart and the Adult Liver Isoform." European Journal of Biochemistry **230** (1): 235-241.

Scheffler, I. (1999). Mitochondria. New York, Wiley-Liss.

Schmidt, C. L. and L. Shaw (2001). "A comprehensive phylogenetic analysis of Rieske and Rieske-type iron-sulfur proteins." Journal of Bioenergetics and Biomembranes **33** (1): 9-26.

Schmidt, T. R., W., M. Goodman and L. I. Grossman (2001). "Evolution of nuclear- and mitochondrial-encoded subunit interaction in cytochrome c oxidase." mol. biol. evol. **18** (4): 563-569.

Sicheritz-Ponten, T., C. G. Kurland and S. G. E. Andersson (1998). "A phylogenetic analysis of the cytochrome b and cytochrome c oxidase I genes supports an origin of mitochondria from within the Rickettsiaceae." Biochimica Et Biophysica Acta-Bioenergetics **1365** (3): 545-551.

Sidell, B. D. and J. R. Hazel (2002). "Triacylglycerol lipase activities in tissues of Antarctic fishes." Polar Biology **25** (7): 517-522.

Sinjorgo, K. M. C., I. Durak, H. L. Dekker, C. M. Edel, T. B. M. Hakvoort, B. F. Vangelder and A. O. Muijsers (1987). "Bovine Cytochrome-C Oxidases, Purified from Heart, Skeletal-Muscle, Liver and Kidney, Differ in the Small Subunits but Show the Same Reaction-Kinetics with Cytochrome-C." Biochimica Et Biophysica Acta **893** (2): 251-258.

Sinjorgo, K. M. C., T. B. M. Hakvoort, A. O. Muijsers, A. W. Schram and J. M. Tager (1988). "Cytochrome-C Oxidase - Organ-Specific Isoenzymes and Deficiencies." Journal of Inherited Metabolic Disease **11**: 202-204.

Smith, P. L., R. I. Krohn, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson and D. C. Klenk (1985). "Measurement of protein using bicinchroninic acid." Analytical Biochemistry **150**: 76-85.

Suarez, R. K., J. R. B. Lighton, B. Joos, S. P. Roberts and J. F. Harrison (1996). "Energy metabolism, enzymatic flux capacities, and metabolic flux rates in flying honeybees." Proceedings of the National Academy of Sciences, USA **93** (22): 12616-12620.

Suarez, R. K., J. F. Staples, J. R. B. Lighton and O. Mathieu-Costello (2000). "Mitochondrial function in flying honeybees (Apis mellifera): Respiratory chain enzymes and electron flow from complex III to oxygen." Journal of Experimental Biology **203** (5): 905-911.

Thibault, M., P. U. Blier and H. Guderley (1997). "Seasonal variation of muscle metabolic organization in rainbow trout (Oncorhynchus mykiss)." Fish Physiology and Biochemistry **16** (2): 139-155.

Wiedemann, F. R., S. Vielhaber, R. Schroder, C. E. Elger and W. S. Kunz (2000). "Evaluation of methods for the determination of mitochondrial respiratory chain enzyme activities in human skeletal muscle samples." Analytical Biochemistry **279** (1): 55-60.

Willett, C. S. and R. S. Burton (2001). "Viability of cytochrome C genotypes depends on cytoplasmic backgrounds in Tigriopus californicus." Evolution **55** (8): 1592-1599.

Wodtke, E. (1981). "Temperature compensation of biological membranes." Biochemica et Biophysica Acta **640**: 710-720.

Zecchinon, L., P. Claverie, T. Collins, S. D'Amico, D. Delille, G. Feller, D. Georlette, E. Gratia, A. Hoyoux, M. A. Meuwis, G. Sonan and C. Gerday (2001). "Did psychrophilic enzymes really win the challenge?" Extremophiles **5** (5): 313-321.