THE LOCAL ADAPTATION OF THE CIRCADIAN CLOCK'S TEMPERATURE COMPENSATION IN *NEUROSPORA*

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ABSTRACT OF THESIS

THE LOCAL ADAPTATION OF THE CIRCADIAN CLOCK'S TEMPERATURE COMPENSATION IN *NEUROSPORA*

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In the current study, we have tested the hypothesis that temperature compensation (TC) of the circadian clock plays a role in local adaptation. To test this hypothesis, we chose strains of *Neurospora* collected from different latitudes; Alaska (high) and Ivory Coast (low). To examine the molecular oscillator of these selected strains, we transformed the natural strains with a translational luciferase reporter of the key clock gene FREQUENCY (FRQ). To examine the developmental overt rhythm, we used the inverted race tube assay. Q_{10} values of the molecular periods and the developmental periods of each strain have been calculated, and used as a quantitative measure of TC. Our data suggest that the molecular oscillators of natural strains collected from different latitudes do not differ from one another, and their Q_{10} values are relatively similar to each other. However, we found that the developmental overt rhythms have different period and Q_{10} values and a period among the strains studied. The periods and Q_{10} values of developmental rhythm are also more variable when compared to those of the molecular rhythm. Taken our results together, we concluded that 1) TC plays a role in adaptation, 2) the adaptation occurred at either the output of the clock or at the coupling mechanism

between the oscillator, and 3) the adaptation occurred at the developmental rhythm rather than the molecular oscillator

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INTRODUCTION

Circadian rhythm is the daily cycle of biological rhythms with a period of about 24hrs, and can be seen in many biological organisms from fungus to mammals including humans. Circadian rhythms play an important role in many aspects of everyday life in many organisms. For instance, in *Neurospora crassa*, it has been shown that the circadian rhythm of this filamentous fungus plays a role in protecting the cell from DNA damage and aids in cell mitosis (Hong, 2014). It has also been shown in rats that the circadian rhythm plays a role in food availability. This means that the circadian rhythm can adjust to the availability of food for the organism. Metabolic efficiency can also be controlled by the circadian rhythm. (Stokkan, 2001). In humans, it has been shown when one's sleep/wake cycle is reversed it can result in many physiological changes: lowered leptin levels, increased glucose levels (despite decreased insulin levels), increased mean arterial pressure, completely reversed the daily cortisol rhythms, and reduced sleep efficiency (Scheer, 2008). Circadian rhythms even play a role in seasonal regulation. For example, the flowering time in a plant model organism *Arabidopsis thaliana* is controlled by many clock- controlled genes (*ccg*). One of such genes is ELF4 which is a *ccg* and also involved in seasonal regulation of flowering (Doyle, 2002).

The authentic circadian rhythms have three main characteristics: First, the period of oscillation of the clock must be approximately 24hrs in constant conditions, second, the rhythm should be entrained (reset) by environmental time cues (i.e. temperature and light), reflecting local time; the repeated and predictable changes in light or temperature must be able to adjust the rhythm exactly 24hrs third, the oscillator must be temperature compensated; the period of the rhythm should be maintained constant

within the physiological temperature range. The first two characteristics have been explained at the molecular level however, there is no known molecular mechanism for temperature compensation yet.

There are four key molecular components in the *Neurospora* circadian clock: FREQUENCY (FRQ), FRQ INTERACTING RNA HELICASE (FRH), WHITE COLLAR-1 (WC-1), WHITE COLLAR-2 (WC-2). There are about a dozen known other molecular components, including kinases/phosphatases (Guo 2010). The interactions among these molecular components are one of the best explanations for the molecular mechanisms of eukaryotic circadian clocks (Brunner, 2008). WC-1 and WC-2, two PAS-domain containing proteins, form the White Collar Complex (WCC). This complex binds to the *frq* gene promoter and activates *frq* transcription. The FRQ protein then binds to FRH to form a complex called the FRQ-FRH complex (FFC). FFC then finds and recruits kinases to phosphorylate the WC proteins that will then lead to the fragmentation of the WCC from the FRQ promoter. This inhibits the transcription of *frq*. Simultaneously, FRQ up-regulates the steady-state levels of WC-1 and WC-2, which forms the positive feedback loop. The hyper-phosphorylated form of FRQ is degraded by 26S proteasome. The Exosome and FFC are used to decompose the *frq* mRNA which forms the negative feedback loop. CSW-1 regulates the accessibility of *frq* promoters (Guo 2010). These series of molecular events takes approximately 24hrs per cycle to occur.

Neurospora is a successful model system for characterizing the eukaryotic circadian clock. There are many advantages in this model system: it has a short sexual life cycle, producing sexual cycles within three weeks, it has publically available genome sequence, whole genome knockout library, and it can be easily manipulated. This allows

more experiments to be performed and more results can be developed over a shorter period of time. The amount of core molecular components that are involved in the circadian clock of *Neurospora* are much smaller when compared to that of other model organisms. This model system is also economical when compared to the cost of performing experiments in other eukaryotic systems. They can be readily re-grown from stock culture and the clonal strain can be reproduced an infinite amount of times. The common themes in molecular structures of the eukaryotic clock makes the Neurospora clock an attractive model organism to study molecular mechanisms of the eukaryotic clock mechanisms (Brunner, 2008).

The idea of TC has been around for many years. It all started in the 4th Century B.C. when Alexander the Great, and scribes noted daily leaf patterns and petal movement of the trees. Also, Androsthenes reported daily rhythms of leaf opening and closing but assumed they were a direct response to environmental stimuli (Dunlap, 2004). In the 1950's, during the early studies of circadian oscillators, Pittendrigh discovered that *Drosophila* emergence rhythm was sufficiently temperature compensated to serve as a useful clock and soon after Pavlidis and Kauzman proposed a biochemical oscillator model for circadian rhythms including activation and inactivation of an enzyme. To maintain TC they stated that there were three requirements necessary: First, rate constant ratios were temperature independent. Second, certain rate constant values assumed to be diffusion controlled to make temperature independent. And third, the product between a rate constant and steady-state concentration of enzyme had to be temperature independent. Years later it was shown that several biological membranes we able to adapt

to temperature by changing the ratio of unsaturated and saturated fats on their surface. This change in ratio was correlated to the temperature change (Ruoff, 1997).

Despite a significant advancement in our genetic and molecular understanding of the circadian clock oscillator, no molecular mechanism of TC has been fully understood (Diernfellner, 2005). Even though biochemical processes are generally dependent upon temperature, the free-running periods of circadian rhythms are very precise and efficiently temperature compensated (Diernfellner, 2005).

It is thought that the two isoforms of FRQ, large FRG (l-FRQ) and small FRQ (s-FRQ), are not required for TC, but they provide a mean to fine-tune period length in response to the ambient temperature (Diernfellner 2007). It has been shown that the level of l-FRQ increases as ambient temperature increases, and the level of s-FRQ decreases with an increase in ambient temperature, and vice-a-versa. This is done by thermosensitive trapping of scanning ribosomes at the upstream opening reading frame (uORF). This leads to reduced translation of the main open reading frame (ORF) and allows adjustment of FRQ levels according to ambient temperature (Diernfellner, 2005). This thermosensitive splicing activity increases as ambient temperature increase resulting in the splicing of intron 6 (I-6) of *frq* RNA increasing. This increase of *frq* RNA, in turn, regulates the ratio of l-FRQ to s-FRQ allowing for *Neurospora* to compensate to the temperature change. Again this splicing regulation of I-6 introns, l-FRG, and s-FRQ are not required for TC but fine tune the ability for *Neurospora* to compensate for temperature fluctuations and changes.

As seen by Mehra *et. al*. (2009), Casein Kinase 2 (CK2) is identified as playing a role in the mechanism underlying circadian TC in *Neurospora*. CK2 was found to be a

key regulator of TC of the *Neurospora* clock. This was done by determining that two long-standing clock mutants, *chrono* and *period-3,* displayed distinct alterations in compensation encode for the β1 and α subunits of CK2, respectively. Phosphorylation-mediated turnover of FRQ is a major determinant of period length in *Neurospora*. By reducing the activity of kinases acting on FRQ, the circadian period length is increased. CK2 exerts its effect on the circadian clock by directly phosphorylating FRQ, and this phosphorylation is compromised by mutations in the β1 and α subunits demonstrated in the *chrono* and *period-3* mutants (Mehra, 2009).

Local adaptation to an organism's environment has been explored in different organisms. As seen by Ellison *et. al.* (2011), population genomics approaches have been used to distinguish local adaptation phenotypic differences in 48 natural isolates of *N. crassa* (Ellison, 2011). The research review by Robinson shows the cold adaptation of Arctic and Antarctic fungi and that the fungus living in the arctic areas of the world can survive the frigid temperatures that the environment can offer (Robinson, 2001). Another example of adaptation is the potato. The ptoato was thought to only be able to be grown in short-day areas. However, it was also that there is a naturally occurring allele that allows potatoes to be cultivated in northern latitudes, long-day areas (Kloosterman, 2013). It was found that this gene belongs to the family of DNA Binding with One Finger (DOF) transcription factors (Yanagisawa, 2004) and regulates tuberization, by acting as a mediator between the circadian clock and the StSP6A mobile tuberization signal (Lee, 2013). There is even some data on circadian clock adaptation to seasonal changes. In Majercak *et. al.*, they show that a thermosensitive splicing event, like that in I6 introns of *s* and *l-frq*, in the 3' region of mRNA from the *period* gene in *Drosophila* plays an

important role in seasonal cold day adaptation (Majercak, 1999). With all the advances in adaptation of organisms and the circadian clock it only makes sense to test the hypothesis that TC of *Neurospora* can naturally adapt to its environment.

One of the technical challenges in studying TC is assaying overt rhythm in extreme temperatures. It is very difficult to use the traditional way of measuring overt rhythm using racetubes. When the temperature becomes either to warm or cold, the organism has trouble growing and the resulting data from the experiment is not an accurate representation of the circadian clock.

As an approach to measure period of an organism at these temperature extremes a luciferase assay may be used to measure the molecular rhythm of the transcription, translation oscillator (TTO). The luciferase assay is a much easier, faster, and accurate way to measure the TTO than the normal race tube analysis. As shown in Larrondo *et. al.*, luciferase reporters reveal clear and sustained luminescence oscillation that can be directly correlate with known FRQ oscillation (Larrondo, 2012). With the help of the automated plate changer and the sensitive luminometer in 96 well format, the luciferase reporter system allows us to study the period of many organisms in a high-throughput manner at a wide variety of temperatures.

The properties of firefly luciferase make it an ideal non-destructive reporter to quantify and image transgene promoters' activity in organisms. Luciferase reporters have been used to make many breakthroughs in circadian rhythms studies in many different model organisms including plants, mammals, and even cyanobacteria. For example, firefly luciferase was used in *Planta* to compare the differences of the effects of protein stability and variation in luminescence within a leaf (variegation) (Leeuwen, 2000).

Inserting luciferase reporters into cyanobacteria has lead to the discovery that 80% of all its genes are clock regulated (Ishiura, 1998). Luciferase reporters have been used to study clock mutants in several different model organisms (Millar, 1995 and Sato, 2006). Luciferase reporters have even been used in the study of stem cells and you can even use luciferase reporters to study the circadian clock in just a single mammalian cell (Legler, 1999). With all these breakthroughs in the field of chronobiology, it only makes sense to use a luciferase reporter to test the TC of *Neurospora.*

In the current study, we tested the hypothesis that TC may play a role in local adaptation. The luciferase reporter can be inserted into different species of *Neurospora* that are grown in entirely different and similar climates around the world. This will allow us to determine and compare TC based off of geographically location. This reporter can also be inserted into a sequenced strain, whose whole genetic make-up is known, as a positive control for transformation and a negative control for TC. For instance, one strain of *N. discreta* (D199) is grown in the arctic environment of Alaska (Shulski, 2007) while another strain (D177) grows in the arid environment of the Ivory Coast (Hoffman, 2003). Comparing these strains TC will help determine if geographical location is a factor in its TC range. In comparison, *N. crassa* is known to be habituated in a temperate region (Jacobson, 2004). Thus, we hypothesized that comparing the TC in two species, *N. crassa* and *N. discreta*, may provide us an insight in local adaptation of TC. These strains can test to see if different species of *Neurospora* have different TC ranges. *N. crassa* Lab strain (FGSC2489) can serve as a negative control for TC because the strain was crossed so many times it actually lost the wild type genotype. This genotype is what would reflect the TC adaption to the local environment that we are expecting to see.

MATERIALS AND METHODS

When performing all experiments there were twelve replicates of each strain made. However, Table 1 shows that not all twelve replicates of each strain had usable data, mainly due to contamination. Depending on the temperature being tested and the strain being used the number of usable replicates varied. There was always at least 4 replicates for each strain.

PPT Extraction and Purification

Use of the bacterial basta resistance gene (*bar*) as a selectable marker in Neurospora was reported (Avalos 1989). Unfortunately, phosphinothricin (PPT, also called glufosinate), the active ingredient in basta, is currently expensive in its pure form. PPT is a principal ingredient in the relatively inexpensive herbicide Finale (Hoechst-Roussel Agri-Vet Inc.) commonly found in lawn and garden stores, but we found that Finale prevents growth of both Bar^+ and Bar^- Neurospora strains. Marty Pall pointed out to us that since PPT is highly soluble in water, a simple extraction may separate the PPT from the non-specific inhibitory ingredients. We found this to be the case. One simple method is to extract Finale twice with an equal volume of 1-butanol, lyophilize the solution, and then dissolve the resulting gel in water (e.g. to half of the original volume). Bioassays indicated that the extracted PPT worked as well as pure PPT and that little if any PPT was lost during the extractions. (Shan 2000)

When using this PPT extract you must add 1.75 mL of the PPT extract to 500 mL of the media you are using. Depending on the strain you may go up to 2.00mL, for example strain FGSC2223 is more immune than other strains so the concentration has to

be increased, but you must never go below 1.75 mL or above 2.0 mL PPT per 500 mL media.

***N. crassa* Transformation with Ignite Selection Protocol**

Grow the strain to be used (Ignite sensitive) in big slants for about 7-14 days 25LL. Make VMFI plates and place in refrigerator until needed. Perform PCR on desired plasmid. Autoclave Top-It-Agar on a liquid cycle for 20 minutes, then place in 60°C water bath. While this is occurring harvest the conidia by adding ice-cold 1M Sorbitol (50ml) to the slant. Shake for about 1 minute in a way that conidia goes into solution. Transfer the content to a 50 ml Falcon tube. Centrifuge for 2min at max rpm. Discard the supernatant. Add 25ml of 1M ice-cold Sorbitol, and re-suspend the conidia. Repeat this one more time. Re-suspend in 10 ml of 1 M ice cold Sorbitol. Quantify by taking 5µl of conidial suspension to 1000µL of Sorbitol in the well, mix well, and Read at OD600. The goal is to get a 5µl aliquot to give 0.5 OD. Adjust volume in order to obtain the right OD. Put 40ul of the spore suspension, mixed well, and transfer to a 2 mm electroporation cuvette. Add 6ul of the PCR product in a pre-chilled Eppendorf. Electroporate, using pulse, at 1500V 600 Ohms and 25µF. Add 1 ml Sorbitol, mix, and add it to a 50ml Falcon containing 3ml of Recovery Media. Shake at 30°C for 1-2 hours to allow for spore recovery. Add to the content of the recovery media spore suspension 8ml of Top-IT-agar. Add to plates containing 25ml of VMFI agar the content of 18. Incubate at 30°C for 3-4 days. Expect about 100 or more colonies. Pick isolated colonies and grow them on Complete PPT Media Slants.

***N. discreta* Transformation with Ignite Selection Protocol**

Grow the strain to be used (Ignite sensitive) in big slants for about 7-14 days 25LL. Make VMFI plates and place in refrigerator until needed. Perform PCR on desired plasmid. Autoclave Top-It-Agar on a liquid cycle for 20 minutes, then place in 60°C water bath. While this is occurring harvest the conidia by adding ice-cold 1M Sorbitol (50ml) to the slant. Shake for about 1 minute in a way that conidia goes into solution. Transfer the content to a 50 ml Falcon tube. Centrifuge for 2min at max rpm. Discard the supernatant. Add 25ml of 1M ice-cold Sorbitol, and re-suspend the conidia. Repeat this one more time. Re-suspend in 10 ml of 1 M ice cold Sorbitol. Quantify by taking 5µl of conidial suspension to 1000µL of Sorbitol in the well, mix well, and Read at OD600. The goal is to get a 5µl aliquot to give 0.750 OD. Adjust volume in order to obtain the right OD. Put 40ul of the spore suspension, mixed well, and transfer to a 2 mm electroporation cuvette. Add 12ul of the PCR product in a pre-chilled Eppendorf. Electroporate, using pulse, at 1500V 600 Ohms and 25µF. Add 1 ml Sorbitol, mix, and add it to a 50ml Falcon containing 3ml of Recovery Media. Shake at 30°C for 1-2 hours to allow for spore recovery. Add to the content of the recovery media spore suspension 8ml of Top-IT-agar. Add to plates containing 25ml of VMFI agar the content of 18. Incubate at 30°C for 3-4 days. Expect about 100 or more colonies. Pick isolated colonies and grow them on Complete PPT Media Slants.

Preparation of Luciferin for In Vitro Bioluminescent Assays

Prepare a 200X Luciferin stock solution (30 mg/ml) in sterile water. Mix gently by inversion until Luciferin is completely dissolved. Use immediately, or aliquot and freeze at -20 °C for future use. (One can either reconstitute the entire 1.0 g of D-Luciferin in 33.3 mL of sterile water to make the 30 mg/mL (200x) stock solution, or reconstitute

the quantity of D-Luciferin necessary for an individual experiment) (Caliper 2008). Add 500µL of 200x stock solution to 25mL of media type. This concentration gives best results in the luminometer.

DNA Easy Plant Mini-Kit Protocol

Place samples in liquid nitrogen for storage during procedure. Add 400uL of AP1 Buffer into each 1.5mL tube. Pour liquid nitrogen into the mortar and pestle. Place the sample in the mortar and pestle and grind carefully (Be sure not to spill the contents of the mortar and pestle over). Using a spatula and place the disrupted fungal material into the corresponding tube, add 4uL of RNase A stock solution and vortex vigorously (Repeat for each sample using clean spatula and mortar and pestle). Incubate for 10 minutes at 65°C. Mix 2-3 times during incubation by inverting the tube. Add 130uL of Buffer P3, mix, and incubate for 5 minutes on ice. Centrifuge lysate for 5 minutes at 14,000rpm (20,000 x g). Pipet lysate (do not disturb the pellet) into QIAshredder Mini spin column (lilac) placed in a 2mL collection tube, centrifuge for 2 minutes at 14,000rpm. Transfer the flow-through from this into a new tube. Add 1.5 volume of Buffer AW1 to the cleared lysate, and mix by pipeting (i.e. if 450uL lysate recovered add 645uL of Buffer AW1). Pipet 650uL from this, including precipitate, into the DNeasy Mini spin column placed in a 2mL collection tube. Centrifuge from 1 minute at 8000rpm, and discard flow-through (re-use collection tube for 13). Repeat this with remaining samples. Discard flow-through and collection tube. Place DNeasy Mini spin column into a new 2mL collection tube, add 500uL Buffer AW2, and centrifuge for 2 minutes at 14,000rpm to dry membrane. Transfer the DNeasy Mini spin column to a 1.5mL tube and pipet 100uLBuffer AE directly on the membrane. Incubate for 5 minutes at room

temperature, and then centrifuge for 1 minute at 8,000rpm. Repeat Step this once (Qiagen 2012). Quantify samples and place them in freezer for later use.

Media procedure and plate set up

The media being used is Luciferin High Glucose Liquid Media (LHGLM). To make LHGLM you add .5mL of D-luciferin into 25mL of High Glucose Liquid media (HGLM). I then add 180uL of the LHGLM media into each well that will be used for testing. After this is complete 20uL of 1:1 conidial suspension is added to the well and stirred using pipette. The wells are then covered with the breathable film and, if the plate is to go in to the stacker, the outside of the film is cut off to prevent sticking.

Racetube Protocol

Low Glucose Racetube media was used for all racetubes. Cover one side of the Racetube with aluminuim foil (make sure nto to rip it). Add 25mL of media to each racetube. Cover the other side of the race tube with aluminium foil (make sure not to rip it) and autoclave for a 30min Fluid cycle. Lay perfectly flate, flame air bubbles out, and let dry overnight. Carefully invert racetubes and let sit for 2 days on drying rack in the Chamber Room. While the Racetubes are drying after they are inverted make HGLM pads of the desired strains. Fill the empty petri dishes with 25mL of High Glucose Liquid Media, UV the HGLM for 15 minutes with lids removed then re-cover. Add 1mL of the HGLM media from pad into the Complete media slant and vortex. Add the vortexed media back into the HGLM pad,cover,, change tips, and ethanol pipet. Repeat this procedure for each strain. Incubate at 25°C in LL condition for 2-3 days. Cut and UV cotton and stuff the non-inoculation end (either bad flip or short end). Punch pads and inoculate, using tweezers, the desired ractube then insert UVed cotton and ethanol

tweezers. Repeat for each desired strain. Put in desired condition and mark either once a day (DD) or twice a day (LD). After experiment is complete take picture using camera and clicker. C lean racetubes when experiment is complete and pictures are aceptable**.**

Luminometer Protocol

Kinetic Study

Open the Softmaz Pro Software. Make sure the luminometer is being read by program. Click file, new experiment. Click the settings button on the new experiment window. Click the kinetic study tab on top of the settings window (not fast kinetic). Make sure luminecense is higlighted in the first section. Go to next section and set your reading time (usually 96hrs) and read time (usually 30min). Scroll to the bottom of the settings window (do not change any other settings). Click the auto read function and set time (Usually same as read time 30min (1800s)). Click the OK button. Click the Read button. Make sure that the plate is being read properly for the first reading. Check on the experiment at least once a day if possible. After the 96hrs are up export the experiment as a XML or text file and repeat the above steps for another 96hrs which will give you 7-8 days of data. Turn iff computer, machines, and chamber when done

End Point Assay

Open the Softmaz Pro Software. Make sure the luminometer is being read by program. Click file, new experiment. Click the settings button on the new experiment window. Click the endpoint assay study tab on top of the settings window. Make sure luminecense is higlighted in the first section. Make sure your integration time is 1sec. Click the OK button. Click the Read button. Wait for it to finish the read of the plate.

Export the data as a XML or text file. Turn off computer, machine, and chamber when done.

Staxmax Protocol

Continuous EndPoint Assasy (Multi-Plate Kinetic Study)

Open the Softmaz Pro Software. Make sure the luminometer is being read by program. Click file, new experiment. Click the Staxmax button on the top menus bar. Click the scripts button in the new window. Either click load script or new:

When starting a new script fill out the top of the forum as specified. First click the Reset button and then click add. First click begin repeat and type in 2000 (just a random number of repeats i choose) the press add. Next click the put plate button then press add. Then press start read button and press add. Then press return plate button and press add. Repeat this for the number of plates you have (i.e. 3 plates will have 3 put plates, 3 start reads, and 3 return plates). Now press the resstack button and type in the numebr of plates the assay will contain then press add. Now press the end repeat button and press add. Save this script so you can use it again.

Now press the SpectraMaxL button on the top right of the screen. In the drop down menu click the bar (should be in a list format):

Now you want top set up where the autosave function will be. Make sure it is set up to save in XML format. Make sure in the main window is set for autosave and XML format. Click OK or Save.

Open up the Staxmax window again, make sure it is your script you need and press run script.When experiment is over save all XML files together use the python program to combine each Endpoint Plate assay into a Multi-plate Kinetic Study.

Re-plating for Bar Nuclei Selection

Original transformants were picked off of the VMFI plate. Colonies are then inoculated on PPT complete media slants. They are then re-inoculated on PPT complete media slants. The original slant is then screened for positive luminescence. All strains with positive luminescence are then re-plated on VMFI plates. Five colonies are then picked from each plate. The picked colonies are inoculated on PPT complete media slants. They are then re-inoculated on PPT complete media slants. The first re-plate picked colony is then screened for luminescence and rhythm. Long term stock is made from the slants with good rhythm and luminescence.

Western Blotting protocol:

Prepare 5ml HGLM liquid cultures in 125ml flasks. Three replicates for one strain. Shake cultures constantly 100RPM. Synchronized clocks by using constant light for 12 hours. Then, the samples are moved to constant dark every 4 hours for 48 hours. The sample is frozen in liquid nitrogen at the end of the 48 hours period. The samples are grinded with mortar and pestle and transfer to 1.5 micro-centrifuge tube. Place 100ul of the non-reducing denaturing extraction buffer:

Mix by gentle inversion. Keep on ice, let sit for 5 minutes and invert again. Centrifuge for 12,000rpm for 15 min. At 4C. Remove supernatant. Measure protein concentration with Bradford protein assay. Prepare a standard curve with known protein concentration of Albumin BSA and then measure concentration of samples (Can also use the Take3 Plate and use the protein quantification program). Aliquot the samples so that 100ug be added to each well. Prepare SDS acrylamide gel- The gels will be in the 4

degree refrigerator. We usually order them. However, you could make it too. The running buffer can also be bought. If bought dilute to 1x with distilled water.

Degas solution by vacuum for 15min. Cassette should be held vertically for gel casting. Add the APS and TEMED to the degassed solution and pour into cassette. Pour smoothly. Overlay slowly with water. Allow gel polymerize for 45min. Rinse the over lay solution with water. Degas gel. Dry the area above the separating gel with filter paper before pouring stacking gel. Place the comb in the cassette and tilt so teeth are at 10 degree angle. Add APS and TEMED. Allow polymerize 30-45min

Pour the solution into the gel container and box. Load your sample. Make sure the Red connects to the Red cord. And black connects to black. Run samples 150V for 65min. Let the dye run to the bottom of the gel. Transfer the proteins to PVDF membrane using Turbotransblotter:

> Pull out the top cassette. Make sure it's clean. Place your PVDF membrane that is label bottom. Use the white roller to press out any air bubbles. Then place your gel on top of the membrane. Then place your Top PVDF membrane. Press out any air bubbles. Lock the cassette into place and place back into the turbotransblotter. Run at 2.5 amps, 25 volts, 13 minutes.

Place membrane in blocking solution 1x PBS , 0.3% Tween 20 5% nonfat milk. Incubate while shaking at room temperature for 1hr or overnight. Replace blocking solution with primary antibody FRQ (1:40 dilution) in 5% milk for one hour. Rinse membrane with PBS-T vigoursly three times. Then wash the membrane with wash buffer for 1 hr, replacing PBS-T solution every 20 minutes. Add secondary antibody (1:250 dilution) in 5% milk Incubate for 1 hr. Rinse membrane with PBS-T vigoursly three

times. Then wash the membrane with wash buffer for 2 hr, replacing PBS-T solution every 20 minutes. Mix detection ECL solutions A and B, in a ratio 40:1, at 0.1ml/cm volume concentration. Drain excess wash buffer from washed membranes and place protein side up on a clean surface. Pipette detection reagent on membrane and incubate for 5mins. Drain off excess detection reagent by holding the membrane gently with forceps and touching the edge against a tissue. On storm image, place the protein side down on scanning bed. Do not let it dry out. Use water to keep moist. Scan using fluorescence/chemifluorescence Blue mode 100 microns at 800V. Save the image as a JPEG using the Storm program. Open the picture file of the membrane in imageJ. Click analyze, select first lane and rectangle out the whole gel area for analysis. Click analyze, plot lane and using the straight line tool connect the peaks so they make a fully closed in shape. Select the wand tool and click the inside of each section that was sectioned off. Click on the table, click select all, and copy and paste into an excel file. Using this data the western data can be graphed and the period can be calculated.

RESULTS

To test the hypothesis that the developmental rhythm TC can adapt to the local environment (high latitude and low latitude), the TC of four different strains and two different species, *N. crassa* and *N. discreta*, were tested at three different temperatures (15°C, 25°C, and 35°C) (Figure 1).

The Alaskan *N. discreta* strain (D199) does not produce conidia at any temperature. This is a growth phenotype that was seen in all replicates. In contrast, the African *N. discreta* (D177) and African *N. crassa* (4825) both show good conidial banding at 25°C. At 35°C however, only the African *N. discreta* still shows good conidial banding, even though it is faint and not as robust as at 25°C. The African *N. crassa* strain still does conidiate but it seems to be spotty and random with no banding seen. Lab Strain *N. crassa*, a sequenced *N. crassa* strain that has been crossed too many times, was used as a transformational control. As seen in Figure 1, Lab Strain *N. crassa* has great conidial banding at 25°C but it does not band at either 15°C or 35°C. At 15°C Lab Strain *N. crassa* seems to just conidiate freely with no observable rhythm. At 35°C Lab Strain *N. crassa* seems to have lower conidiation, if it conidates at all.

The normal developmental overt rhythm period is approximately 22hrs. Figure 2 shows the representative of a typical trace of *replicate* strains ran on racetubes. It can be seen by Figure 2 that the racetube images are correctly converted into graphs that are able to show the rhythm of the conidiation. Figure 2 also shows the conidial density of each strain relative to each other. Figure 3 shows the average developmental period of Africa *N. discreta*, Africa *N. crassa*, and Lab Strain *N. crassa*. The error bars were calculated

using the standard error. Table 3 shows the average Q_{10} values of each strain with, standard error, where N=4. For a strain to be temperature compensated the Q_{10} values of that strain must be between 0.8 and 1.2. The closer the Q_{10} value is to 1.0, the better compensated the strain is. In Table 3 you can see that at 15°C the Alaskan N. discreta strain and the Lab Strain *N. crassa* were both temperature compensated, with a Q_{10} value of 0.961 and 0.919 respectively. At 35°C, Table 3 shows that the Alaskan N. discreta and African N. discreta strains were temperature compensated, with a Q_{10} value of 0.989 and 1.066 respectively. Figure 4 shows the calculated Q_{10} values of the strains relative to each other. In Figure 4A, you can see that the African strain has a better compensation at Q_{10}^{W} then at Q_{10}^{C} with a p-value of 0.003. Figure 4 also shows that there is no calculation of TC for the Alaskan N. discreta strain at either temperature. You can see the TC of African *N. crassa* TC at either temperature is not significant (P-value of 0.175). Figure 4 shows the TC of the African *N. discreta* strain at Q_{10}^{W} which is better compensated than that of the African *N. crassa* strain (P-value of 0.011). Figure 4 also shows that you could only calculate TC at one temperature, Q_{10}^{W}, for the Lab Strain *N. crassa* and it was not compensated.

To test the hypothesis that the molecular TC can adapt to the local environment (high latitude and low latitude), the molecular TC of four different strains and two different species (*N. crassa* and *N. discreta*) of *Neurospora* were tested at three different temperatures (15°C, 25°C, and 35°C). When performing the experiments there were twelve replicates of each strain made for every experiment. However, Table 4 shows that not all twelve replicates of each strain had usable data. Depending on the temperature, the

strain being used, and if there was contamination present, the number of usable replicates varied. There were always at least four usable replicates for each strain.

Luminometer experiments were performed on the four chosen strain at the three chosen temperatures. Figure 5 shows the graphical representation of the period of each of these strains at the chosen temperatures after Chrono3 conversion was performed. The graphs are separated by of species to better show the robustness of the graphs. Each line is the representative of a typical trace of replicate strains ran in the lumniometer. It can be seen in Figure 5 that all the strains have a visible rhythm even though there is a difference in the luminescence value.

After the luminometer experiment was completed the excel sheets were converted into Chrono and analyzed using Chrono3 periodogram algorithm. Table 5 shows the average period of each strain at the three different temperatures including the standard error (STER). Figure 6 shows the graphical representation of the average periods including the standard error. This graph shows that the period is typically well conserved over the temperature ranges except for strain Lab Strain *N. crassa,* in which the variation between temperatures is very high.

Table 6 shows the average Q_{10} values of each strain with standard error, where N=4. In Table 6 you can see that at 15°C the Alaskan *N. discreta*, African *N. discreta*, and African *N. crassa* were temperature compensated, with a Q_{10} value of 0.951, 0.999, and 0.998 respectively. At 35°C Table 3 shows that the Alaskan *N. discreta*, African *N. discreta*, African *N. crassa*, and the sequenced strain were temperature compensated, with a Q_{10} value of 1.026, 0.985, 1.037, and 0.81 respectively. Figure 7 shows that

calculated Q_{10} Values of the strains tested. In Figure 7, you can compare the TC of both the *N. discreta* strains to one another. You can see that there is no difference in TC of neither African nor Alaskan *N. discreta* at Q_{10}^{W} or Q_{10}^{C} with a p-value of 0.718 and 0.090 respectively. You can see compare the TC of African *N. crassa* to Alaskan *N. discreta*. It again shows no significant difference in TC with a P-value of 0.541 and 0.090 respectively. This allows you to also compare the TC of low latitude African. Yet again, it shows no difference in the TC of African *N. crassa* and *N. discreta* (P-value of 0.718 and 0.541The next comparison seen is that of the African *N. crassa* and Lab Strain *N. crassa*. As stated previously, there is no significant difference in TC at either temperature for the *N. crassa* strains (P-value of 0.541 and 0.753 respectively). However, it was recorded that Lab Strain *N. crassa* did not have TC at Q_{10}^{C} and was barely compensated at Q_{10}^{W}. For this reason Lab Strain *N. crassa* is a great negative control for not only TC but to also believe that the data is an accurate representation of the clock. However, the luminometer periods were all extremely close together, with some variation, so it was still hard to conclude that this was actually representing the molecular rhythm of the strains or was it just giving off a response to something else.

To test the hypothesis that the luminometer was a correct and accurate representation of the molecular data a western blotting analysis was required, only one strain was chosen as a representative transformation strain. 4825TL (African *N. crassa*) was the chosen representative strain because it is a *N.* crassa strain, which is what the antibody was made for, and shares the same phenotype and molecular rhythm as the African *N. discreta*. In other papers the western blot analysis reveals a 22hr period. Since our luminometer data was showing a 24hr period it was thought that it was not correctly

representing the molecular clock. For this reason the western analysis was performed. Figure 8 shows the membrane of the 12 time points that were tested, every 4hrs for 48hrs, with the loading control bans. Figure 9 shows the relative FRQ expression graphed over time. From this expression you can see that the first peak of FRQ protein is at CT16 and the next peak is at CT40. This means that the calculated period of the western blot is 24hrs.

Now that it has been proven that the luminometer is an accurate representation of the molecular clock, the TC of the both molecular and developmental rhythms were next compared to see if the traditional racetube method was a accurate representation of the circadian clock, during TC, and to see which was more accurate, the racetube method or luminometer luciferase reporter method. Figure 10 shows the graphical comparison of the developmental period, racetubes, to that of the molecular period, luminometer luciferase reporter. In Figure 10A, it can be seen that there is a significant difference in the calculated period at 15°C of the Alaskan *N. discreta* and the African *N. crassa* (P-Value of 0.006 and 0.026 respectively), however there is no significant difference in the calculated period of African *N. discreta* and Lab Strain *N. crassa*, the sequenced strain, (P-value of 0.300 and 0.547 respectively). In Figure 10B it can be seen that there is a significant difference in the calculated period at 25°C of Alaskan *N. discreta*, African *N. discreta*, African *N. crassa*, and Lab Strain *N. crassa* (P-values of 0.041, 0.013, 0.047, and 0.027 respectively). In Figure 10C, it can be seen that there is a significant difference in calculated period at 35°C for Alaskan *N. discreta* and African *N. crassa* (P-value of 0.036 and 0.034 respectively), however there is no significant difference in the calculated

period of African *N. discreta* and Lab Strain *N. crassa* (P-Value 0.101 and 0.500 respectively).

The calculated Q_{10} values were then compared to determine if TC of the molecular clock is better determined using racetubes, developmental rhythm, or luminometer luciferase reporter, molecular rhythm. Figure 11 shows the graphical comparison of the developmental, racetubes, and Molecular, luminometer luciferase reporter, Q_{10} values. In Figure 11A it can be seen that there is a significant difference in calculated Q_{10} values at 15°C for African *N. discreta*, African *N. crassa*, and Lab Strain *N. crassa* (P-Values of 0.002, 0.023, and 0.026 respectively) however, there was no significant difference of the Alaskan *N. discreta* strain (P-Value of 0.743). In Figure 11B it can be seen that there is a only a significant difference in calculated Q_{10} value at 35°C of the African *N. crassa* strain (P-Value of 0.023) with the other strains having no significant difference in their calculated Q_{10} value.

DISCUSSION

The goal of this study was to test if TC is able to adapt to its local climate. Our data suggests that the TC of the African *N. discreta* strain is better compensated at the 35°C temperature than that of the 15°C. Its Q_{10} value is 1.066, Table 3, with very little standard error making it the best compensated strain at 35°C. One of our favorite interpretations is to look at the environment that the African *N. discreta* strain lives in. The average temperature of the Ivory Coast is only 27°C however, for the summer months the temperature averages anywhere from 24 - 34°C. This temperature fluctuation means that this strain has to be able to adapt to the changes in warmer temperatures but

has no reason to adapt to the cold, which is why its Q_{10} value is below 0.8. It was expected that the African *N. crassa* strain to match that of the African *N. discreta* strain however this was not entirely true. Both of these strains had bad compensation at 15°C, when compared to the other strains, as expected, however the African *N. crassa* also had bad compensation at 35°C. Now both the African *N. crassa* and *N. discreta* strains were collected from the same location, actually about 1° apart from each other. It can be said that they have the close to the same, if not the exactly the same, environment, but their TC differs. This may be explainable due to the fact that they are different species of *Neurospora* and, with no known TC mechanism known, may have a different strengths of developmental TC. However this has not yet been proven. The Alaskan *N. discreta* strain was expected to be better compensated at 15°C than 35°C however, this is not true. It is known that the conidiation and growth of the Alaskan *N. discreta* strain in racetubes was poor, in fact it does not produce any usable period data at all. This was one of the driving factors to insert the translation FRQ reporter into the genome and measure the molecular clocks rhythm and use that to calculate Q_{10} values.

In contrast to the racetubes the luminometer period variation is rather low, as can be seen when comparing Table 2 to Table 5 and Figure 3 and Figure 6. In Table 2 it can see that the Standard Error of the period can be very high and varies with temperature however, as shown in Table 5, the Standard Error is not that high, excluding Lab Strain *N. crassa* (which is not temperature compensated), and does not vary between different temperatures. This trend continues to be shown when comparing Table 3 to Table 6 and Figure 7 to Figure 11. When comparing the Q_{10} values, Table 3 and 6, it can be seen that the amount overall temperature compensated strains, with useable data, is limited to a

total of 1 strain at each temperature in racetubes. However, the luminometer shows that three of the four strains were temperature compensated at both temperatures (the only one not compensated was Lab Strain *N. crassa* which does not have TC). Furthermore if you were to compare the TC of the same strains at different temperature it can be seen that the Q_{10} variation is very high in racetubes but not in the luminometer. In fact the Q_{10} values of all strains, which were temperature compensated, are very similar (all hovering around 1.0) meaning that the luminometer shows very constant rhythm of the clock with no real change as a result of temperature.

This experimental data shows that the luminometer is not only a better representation of the TTO but is also a better representation of the TC of the TTO. The luminometer gives consistent molecular rhythm with consistent period data even at different temperatures. When compared to the developmental rhythm the period at TC is more stable and less susceptible to change. The data represented here would suggest that the local adaptation of TC occurred at the physiological, developmental rhythm, level rather than the molecular, TTO, level. This gives rise to the idea that the racetube experiments are not showing only the TTO but another clock mechanism that is highly affected by temperature. With the new discoveries that the circadian clock NAD^+ cycle drives mitochondrial oxidative metabolism in mice (Peek 2013), it may even be possible that there is a metabolic oscillator, which is highly effected by temperature, which communicates with the TTO and gives the rhythm seen in racetubes. This would explain not only the decoupling we see in TC but also the natural decoupling that we have noticed at 25°C. Figure 12 is a visual representation of what the decoupling of the molecular oscillator and the developmental rhythm that we have seen. This decoupling of

molecular rhythm, luminometer, and developmental overt rhythm, racetubes, at 25°C adds more information on the fact that racetubes may not be a accurate representation of the molecular oscillator, but a representation of the molecular oscillator in cooperation with something else to give the rhythm seen in racetubes.

CONCLUDING STATEMENT

In conclusion, experimental data demonstrate that racetubes are not an accurate representation of the TTO, as thought, and the luminometer is a better experimental representation of the effects on the TTO. The luciferase reporters give a more accurate representation of the TTO not only at different temperatures but at the ideal growing temperature of 25°C. In addition there is a natural decoupling occurrence not only at the different temperatures here but also at the ideal growing conditions. This suggests that there is another oscillator that cooperates with the TTO, maybe a metabolic oscillator, to give the phenotypes we see in racetubes. This experimental data also demonstrates that the adaption of the TC to the local environment occurs at the physiological, developmental overt rhythm, level rather than the molecular level. Suggesting that to study changes in the molecular oscillator of *Neurospora* it is better to use a luminometer measuring luciferase reporter activity rather than the traditional racetube experiment method.

FIGURES

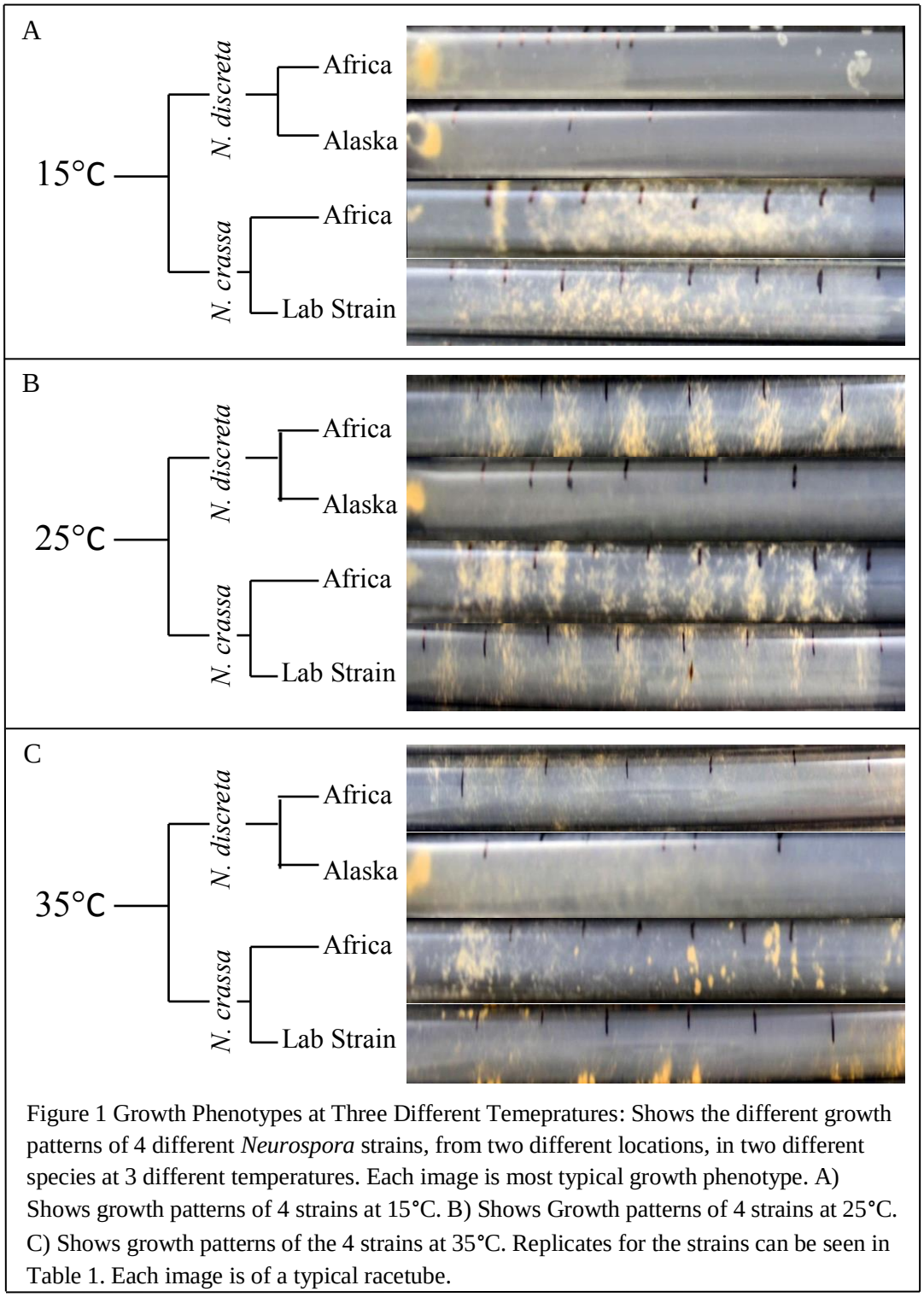


Figure 1 Growth Phenotypes at Three Different Temepratures: Shows the different growth patterns of 4 different *Neurospora* strains, from two different locations, in two different species at 3 different temperatures. Each image is most typical growth phenotype. A) Shows growth patterns of 4 strains at 15°C. B) Shows Growth patterns of 4 strains at 25°C. C) Shows growth patterns of the 4 strains at 35°C. Replicates for the strains can be seen in Table 1. Each image is of a typical racetube.

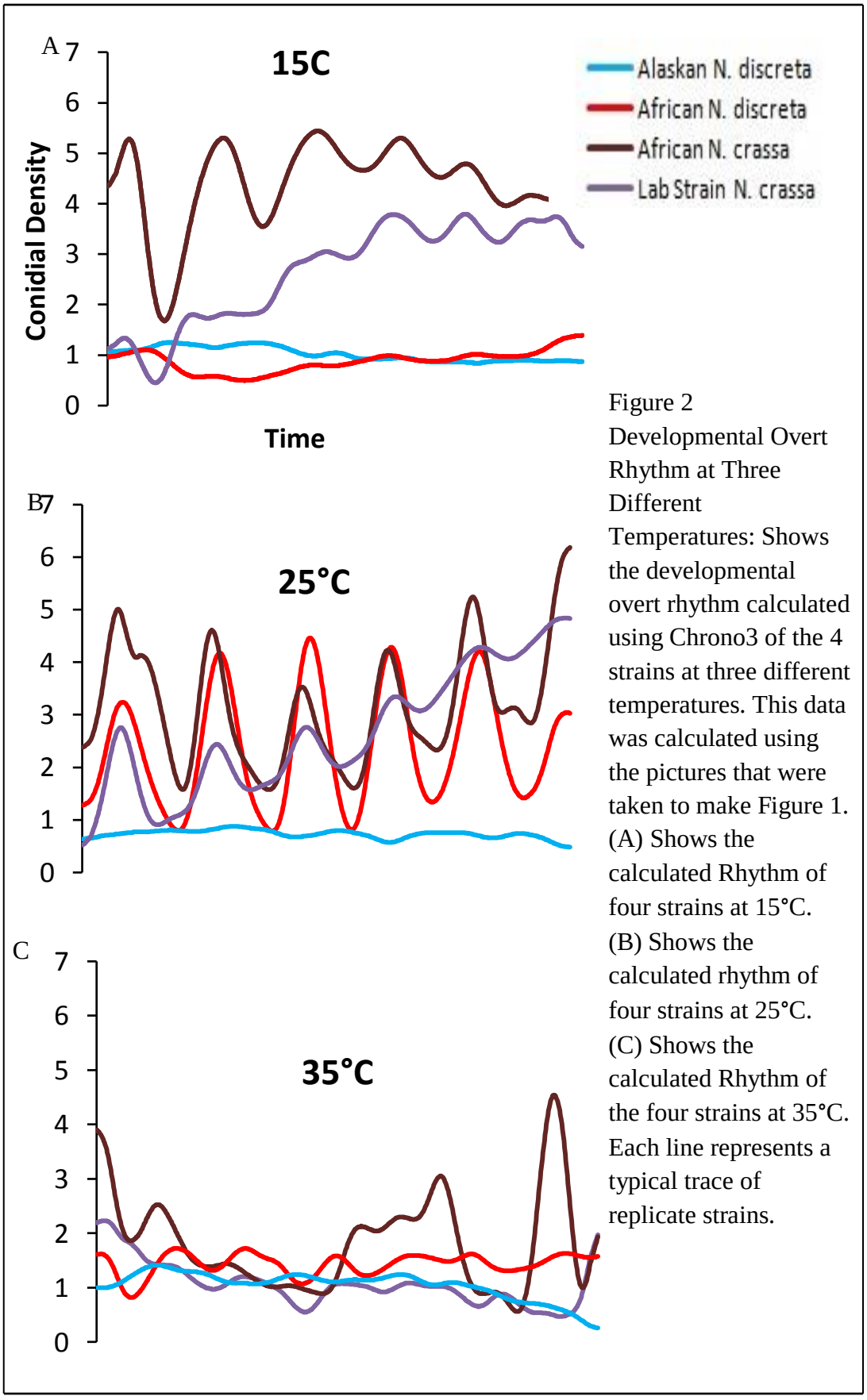


Figure 2 Developmental Overt Rhythm at Three Different Temperatures: Shows the developmental overt rhythm calculated using Chrono3 of the 4 strains at three different temperatures. This data was calculated using the pictures that were taken to make Figure 1. (A) Shows the calculated Rhythm of four strains at 15°C. (B) Shows the calculated rhythm of four strains at 25°C. (C) Shows the calculated Rhythm of the four strains at 35°C. Each line represents a typical trace of replicate strains.

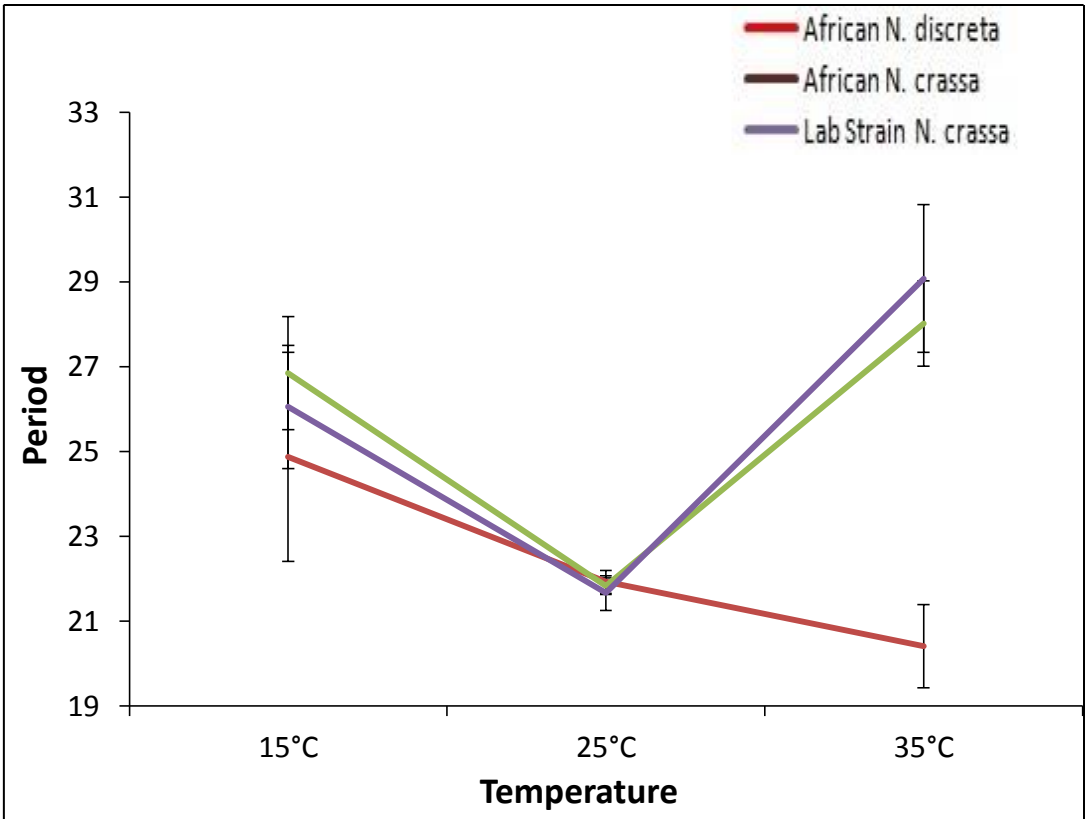


Figure 3 Calculated Developmental Period at Three Temperatures: Shows the average developmental period of three different *Neurospora* strains, from two different locations, at three different temperatures.

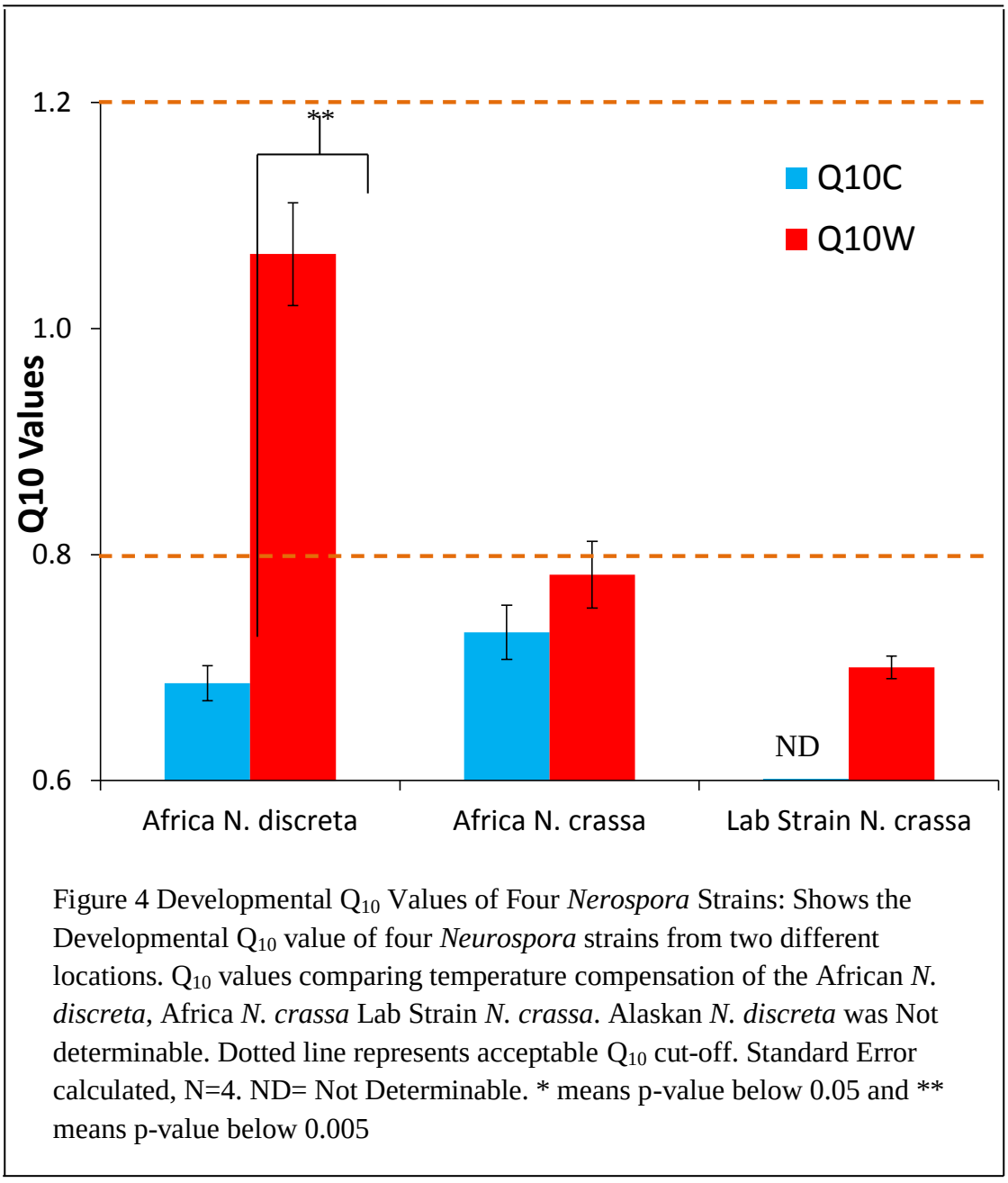


Figure 4 Developmental Q_{10} Values of Four *Nerospora* Strains: Shows the Developmental Q_{10} value of four *Neurospora* strains from two different locations. Q_{10} values comparing temperature compensation of the African *N. discreta*, Africa *N. crassa* Lab Strain *N. crassa*. Alaskan *N. discreta* was Not determinable. Dotted line represents acceptable Q_{10} cut-off. Standard Error calculated, N=4. ND= Not Determinable. * means p-value below 0.05 and ** means p-value below 0.005

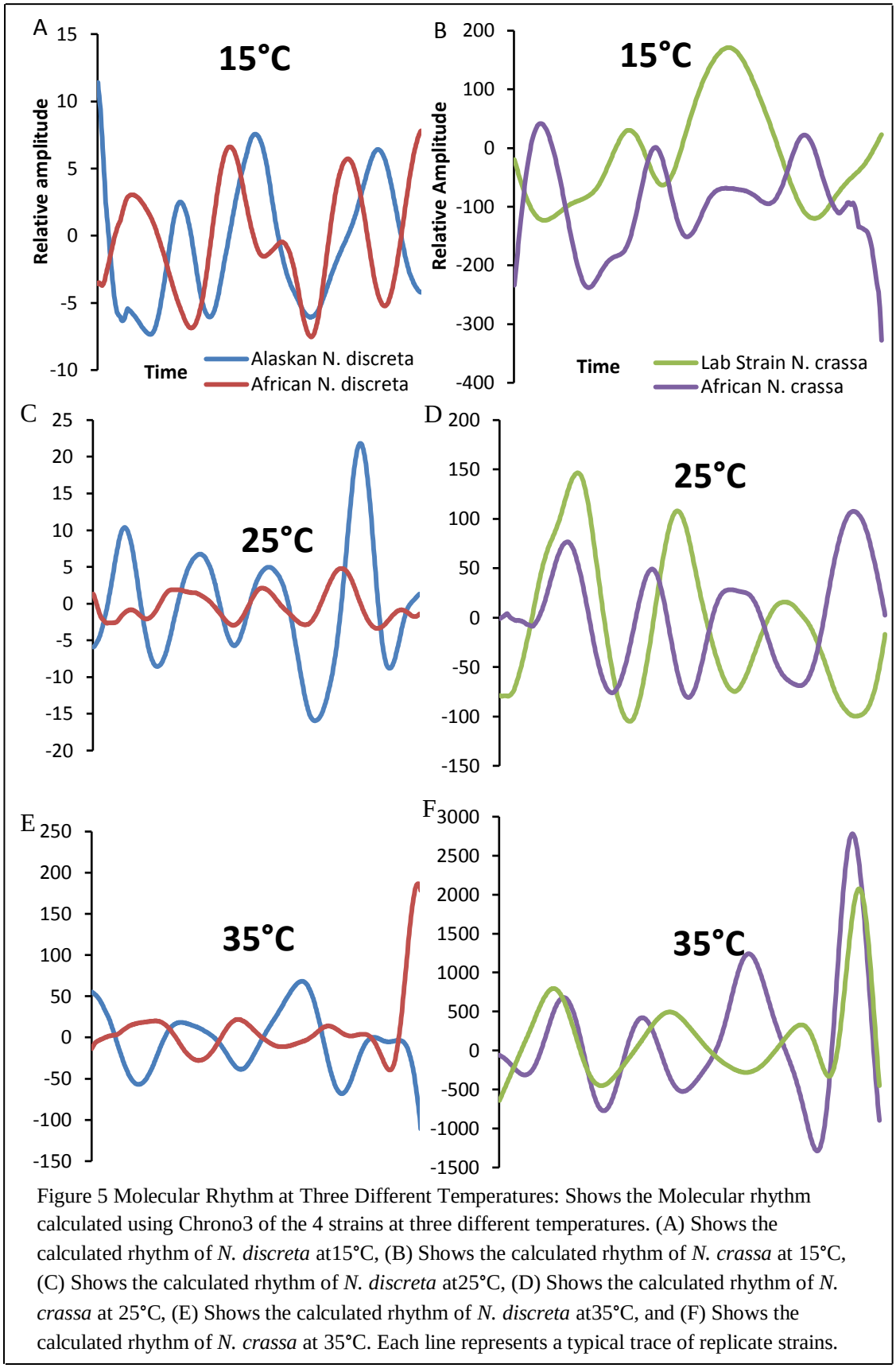


Figure 5 Molecular Rhythm at Three Different Temperatures: Shows the Molecular rhythm calculated using Chrono3 of the 4 strains at three different temperatures. (A) Shows the calculated rhythm of *N. discreta* at15°C, (B) Shows the calculated rhythm of *N. crassa* at 15°C, (C) Shows the calculated rhythm of *N. discreta* at25°C, (D) Shows the calculated rhythm of *N. crassa* at 25°C, (E) Shows the calculated rhythm of *N. discreta* at35°C, and (F) Shows the calculated rhythm of *N. crassa* at 35°C. Each line represents a typical trace of replicate strains.

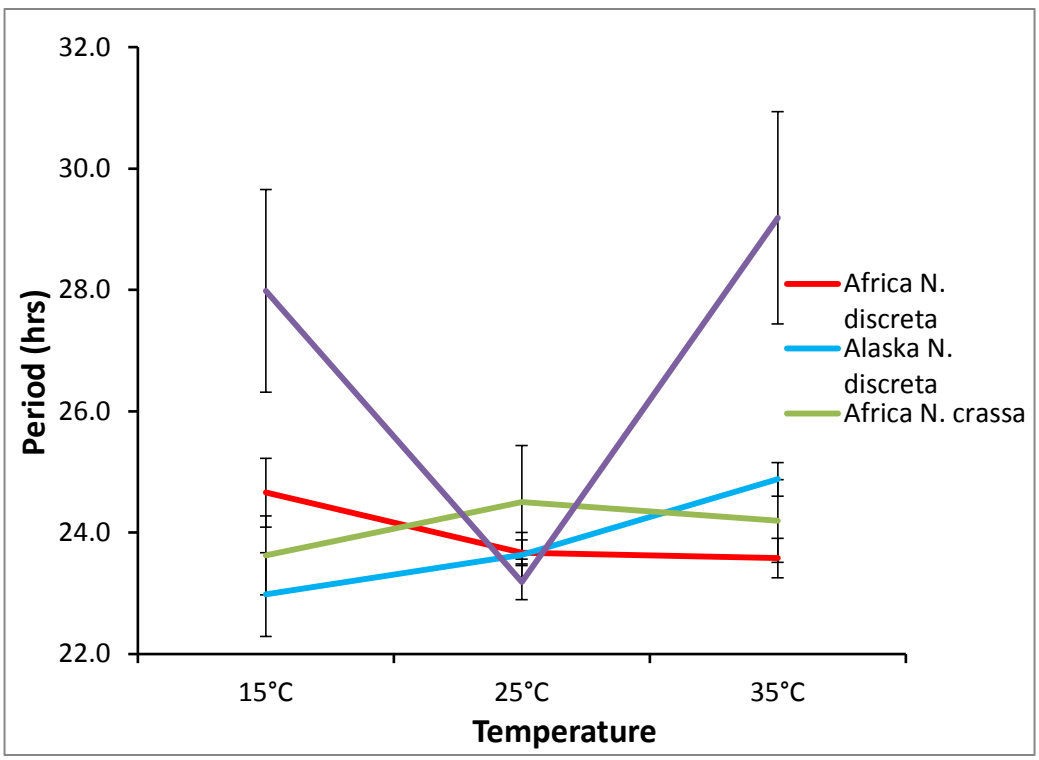


Figure 6 Calculated Molecular Period at Three Different Temperatures: Shows the average Molecular period of four different *Neurospora* strains, from two different locations, at three different temperatures.

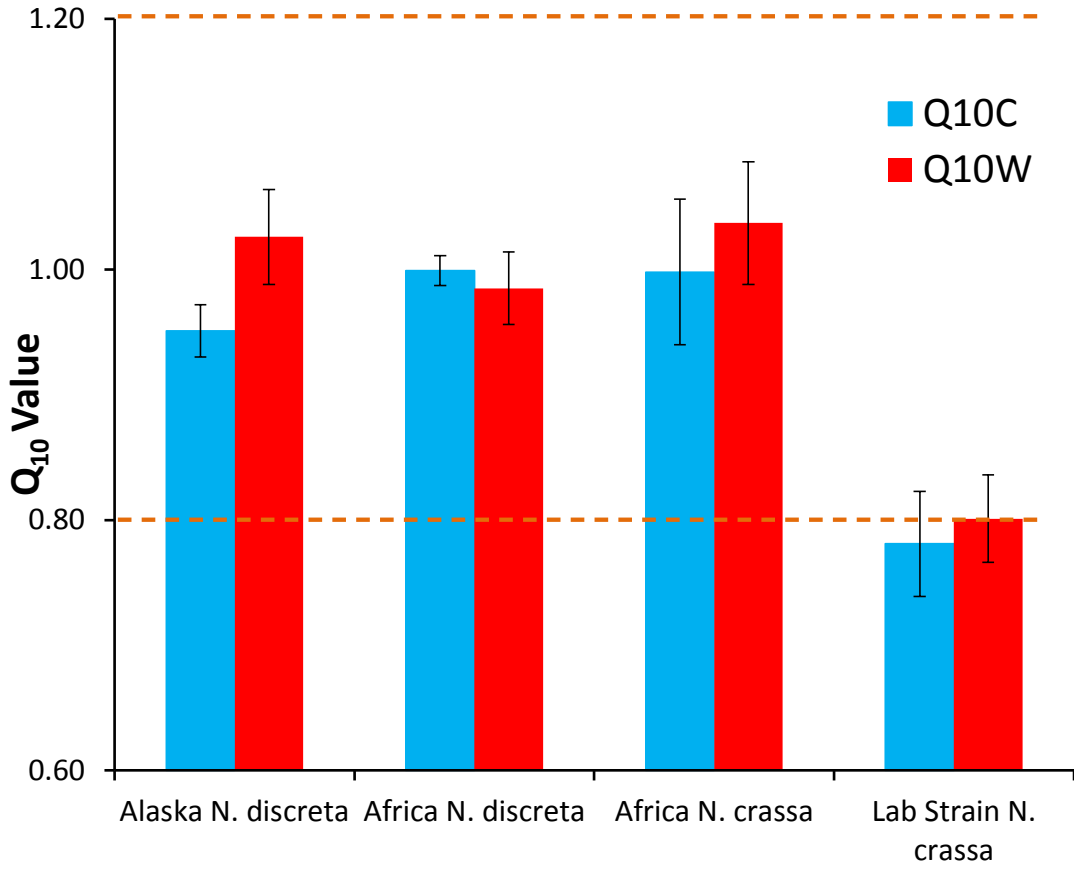
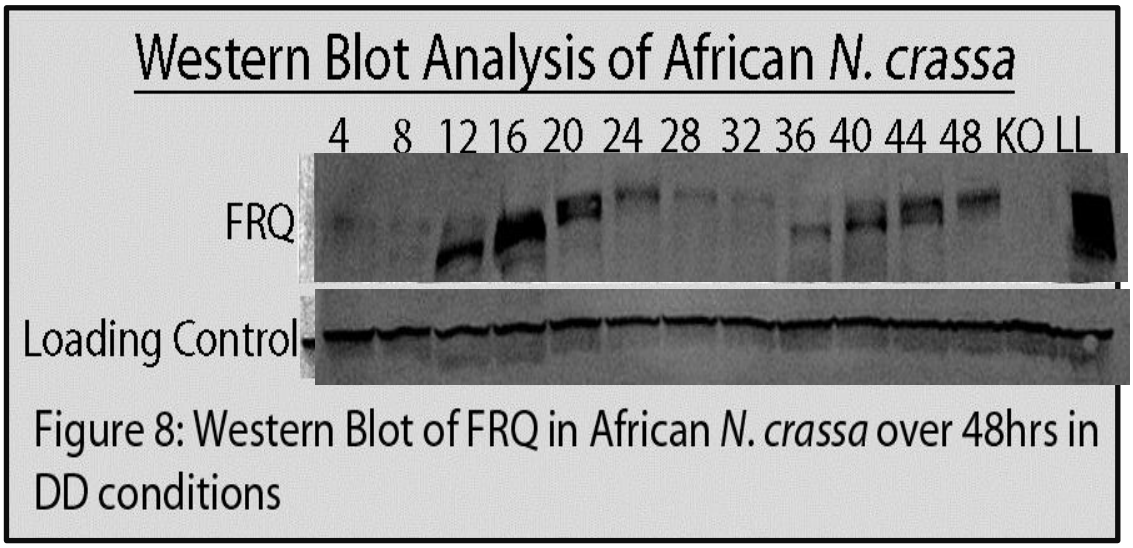


Figure 7 Molecular Q_{10} Values of four *Neurospora* strains: Shows the molecular oscillator's Q_{10} value of four *Neurospora* strains from two different locations. These Q_{10} values are comparing temperature compensation of the African *N. discreta*, Africa *N. crassa* Lab Strain *N. crassa*. Dotted line represents acceptable Q_{10} cut-off. Standard Error calculated, N=4

Figure 8: Western Blot Analysis of African *N. crassa*



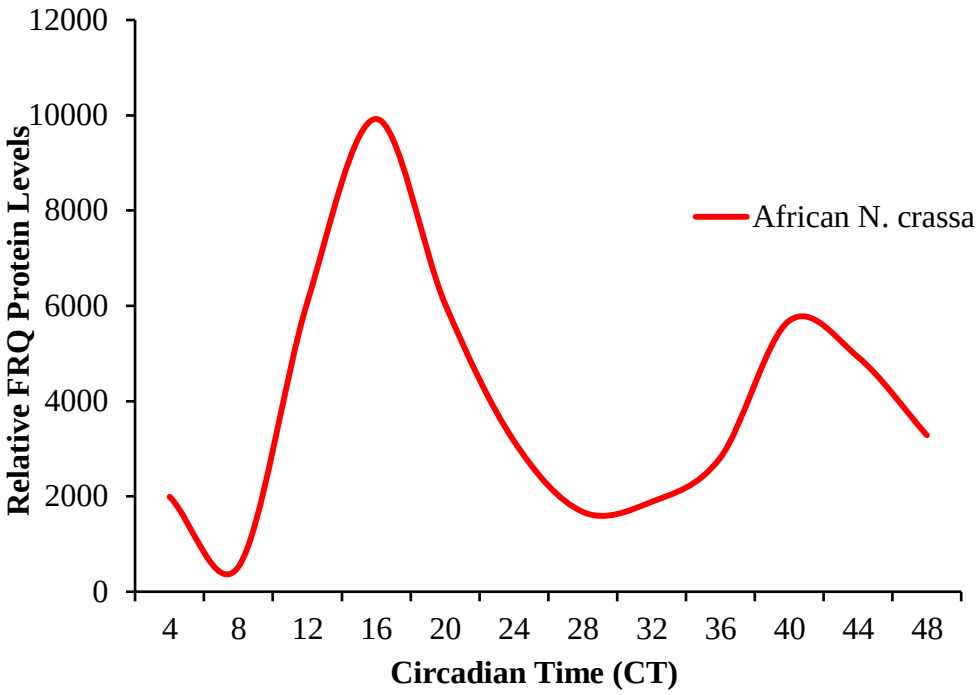


Figure 9 African *N. crassa* Western Blot Analysis: Shows the western blot analysis of African *N. crassa* over 48hrs. Peaks are at CT16 and CT40.

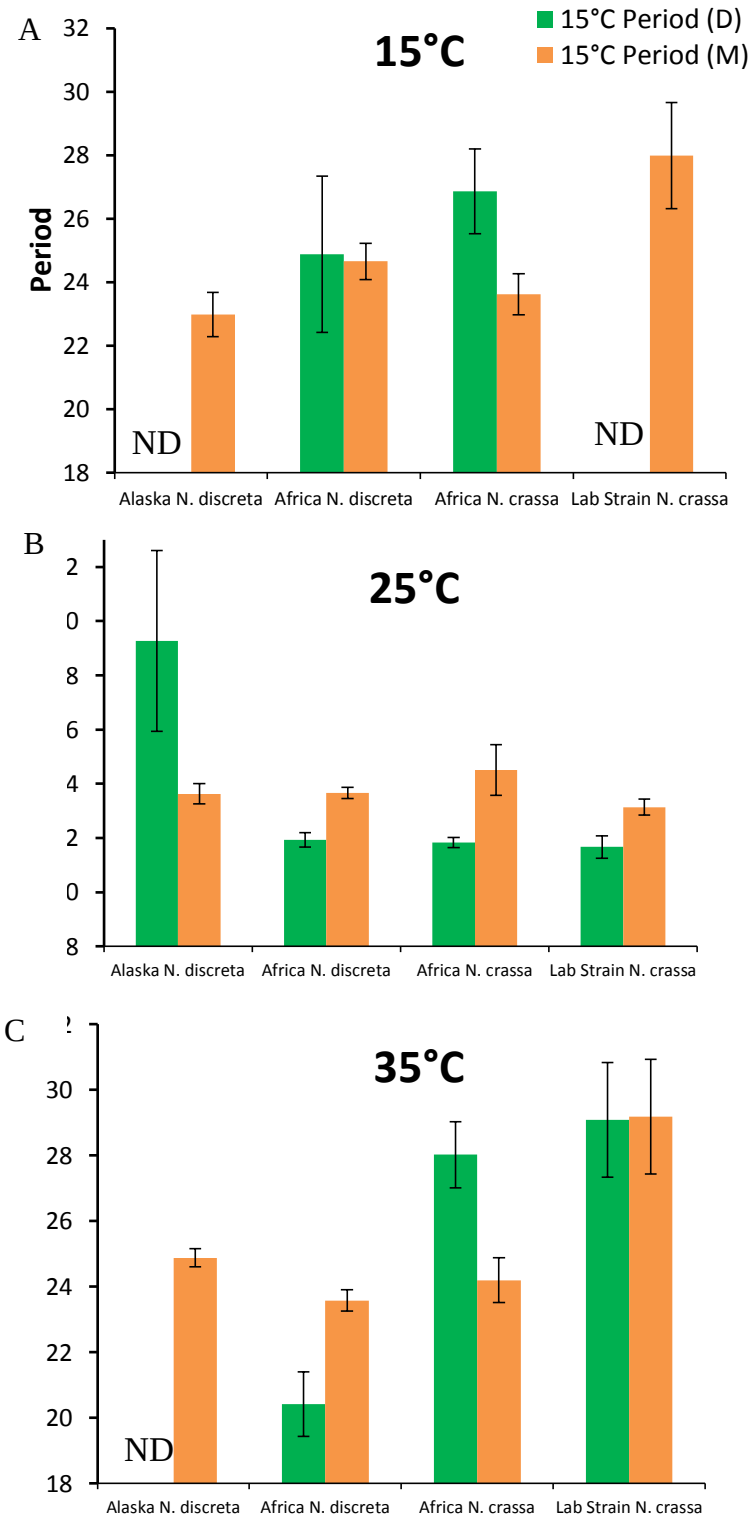


Figure 10 Comparison of Developmental (D) and Molecular (M) Period : Shows the calculated period at all three temperatures tested. (A) The calculated developmental and molecular period at 15°C. (B) The calculated developmental and molecular period at 25°C. (C) The calculated developmental and molecular period at 35°C.

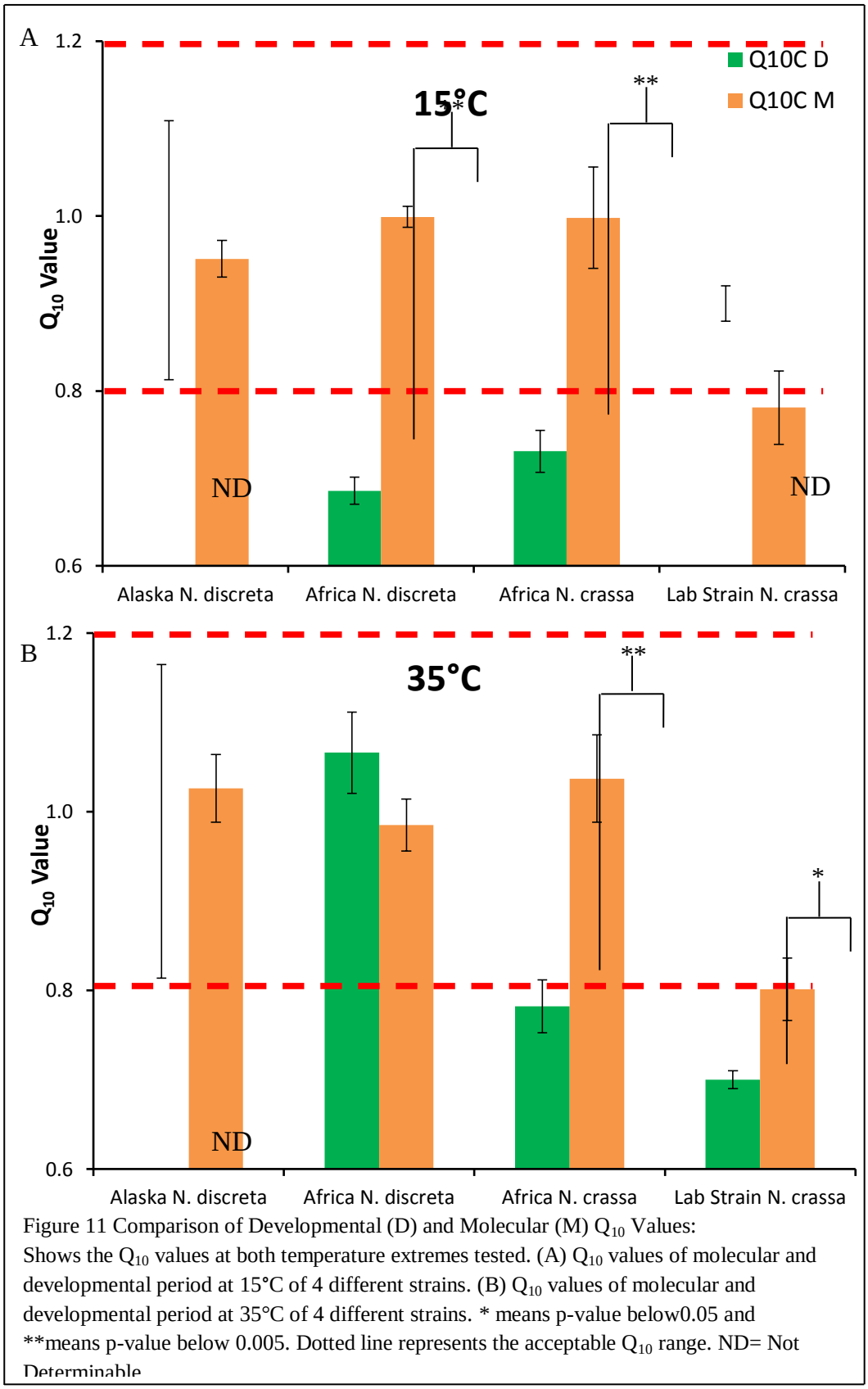


Figure 11 Comparison of Developmental (D) and Molecular (M) Q_{10} Values:
Shows the Q_{10} values at both temperature extremes tested. (A) Q_{10} values of molecular and developmental period at 15°C of 4 different strains. (B) Q_{10} values of molecular and developmental period at 35°C of 4 different strains. * means p-value below0.05 and **means p-value below 0.005. Dotted line represents the acceptable Q_{10} range. ND= Not Determinable

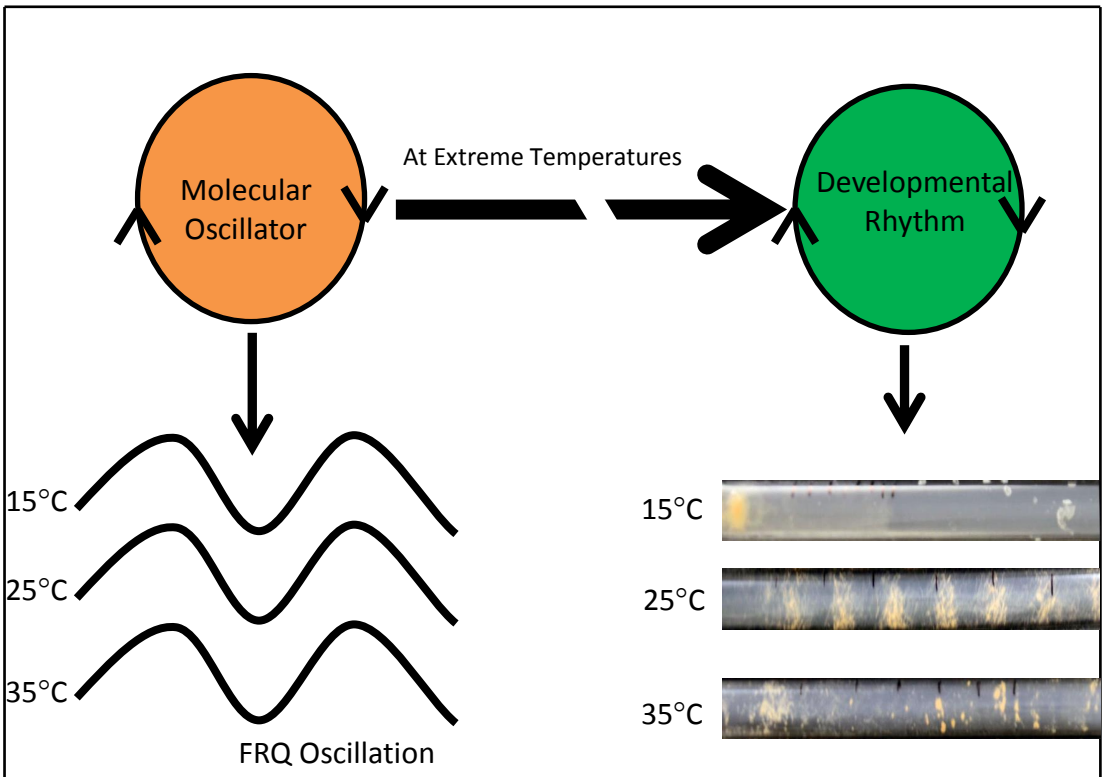


Figure 12 Visual Representation of De-Coupling: Shows that there is a communication between the molecular oscillator and what we seen in the developmental rhythm during laboratory conditions. But, at extreme temperatures, there is a decoupling seen between the molecular oscillator and the developmental rhythm.

TABLES

Table 1: Number of Racetube Replicates per Strain

Strain	Replicates @15	Replicates @25	Replicates @35
Alaska *N. discreta*	12	12	7
Africa *N. discreta*	12	10	7
Africa *N. crassa*	12	9	4
Lab Strain *N. crassa*	9	7	7

Shows the number of racetube replicates used for period and Q_{10} calculations

Table 2: Average Developmental Period

Strain	15°C Period	15°C STER	25°C Period	25°C STER	35°C Period	35°C STER
Alaska *N. discreta*	ND	ND	ND	ND	ND	ND
Africa N. discreta	24.877	2.465	21.933	0.273	20.414	0.983
Africa *N. crassa*	26.856	1.333	21.83	0.193	28.022	1.011
Lab Strain *N. crassa*	ND	ND	21.667	0.408	29.084	1.747

Shows the calculated average period of each strain. STER= standard error. N values are taken from table 1. ND= Not Determinable

Table 3: Average Q_{10} Values

Strain	15-25C Q10 value	STER 15-25C	25-35C Q10 value	STER 25-35C
Alaska *N. discreta*	ND	ND	ND	ND
Africa N. discreta	0.686	0.016	1.066*	0.046
Africa *N. crassa*	0.731	0.024	0.782	0.030
Lab Strain *N. crassa*	ND	ND	0.706	0.010

Shows the Q_{10} values of the molecular rhythm with standard error (STER) where N=4. * Means strain was temperature compensated.

Table 4: Number of Molecular Replicates per Strain

Strain	Replicates @15°C	Replicates @25°C	Replicates @35°C
Alaska *N. discreta*	8	10	8
Africa *N. discreta*	9	9	7
Africa *N. crassa*	4	12	7
Lab Strain *N. crassa*	11	12	8

Shows the number of replicates used for period calculations

Table 5: Average Molecular Period

Strain	15°C Period	15°C STER	25°C Period	25°C STER	35°C Period	35°C STER
Alaska *N. discreta*	22.980	0.691	23.633	0.366	24.879	0.276
Africa *N. discreta*	24.685	0.568	23.667	0.212	23.579	0.323
Africa *N. crassa*	23.622	0.652	24.501	0.936	24.194	0.681
Lab Strain *N. crassa*	27.985	1.671	23.139	0.291	29.187	1.750

Shows the calculated average period of each strain. STER= standard error. N values are taken from table 1. ND= Not Determinable

Table 6: Average Molecular Q_{10} Values

Q10 values				
Strain	15-25°C Q10 value	STER 15-25°C	25-35°C Q10 value	STER 15-25°C
Alaska *N. discreta*	0.951*	0.021	1.026*	0.038
Africa *N. discreta*	0.999*	0.012	0.985*	0.029
Africa *N. crassa*	0.998*	0.058	1.037*	0.049
Lab Strain *N. crassa*	0.781	0.042	0.801*	0.035

Shows the Q_{10} values of the molecular rhythm with standard error (STER) where N=4. * Means strain was temperature compensated.

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