# Introduction

**Climate change has begun to shift environmental conditions away from historic thermal regimes that populations evolved under**. As climate continues to shift species may struggle to keep pace1.

Evolutionary processes have previously been ignored when projecting species responses to climate change due to the thought that they were too slow to influence measurable demographic effects 2. However, species may overcome this obstacle via large effective population sizes and fecundity rates, whereby (if the necessary genetic variation is available) strong selection pressures could produce sizeable changes in allele frequency within a single generation/cohort 2. Therefore, important to consider evolutionary process that will influence past and future populations responses to climate change. **This information should come later maybe even just have in discussion??**

**Local adaptation occurs within metapopulations when native genotypes are better adapted to local environment conditions than foreign genotypes** 3–5. Through gene x environment interactions, local adaptation may arise in spatially heterogenous environments if divergent selection can overcome the homogenizing effects of gene flow and (temporal) instability in selective forces 3,6–10. Isolated populations are particularly susceptible to local adaptation... Metapopulations may therefore be comprised of a mosaic of locally adapted populations that have evolved optimized traits suited to local environments.

**Thermal conditions across latitudinal gradients can shaped the fitness landscape via locally adapted traits.** ~~Local adaptation typically thought of \_\_\_\_\_, but can also be in the form of thermal tolerances (Aitkens and Travis 2010). The pervasive nature of temperature at various biological levels (e.g. cellular biochemistry, physiological processes), particularly among ectotherms, suggests that it can impose strong divergent selection pressures on populations~~ ~~11~~~~.~~ Evidence of temperature-dependent clines between populations is widespread, having been demonstrated in plants 12,13, copepods11,14,15, *Daphnia*16, *Drosophila* (review 17), porcelain crabs18, and intertidal snails19,20 (also see Sanford and Kelly (2011)21 for review in marine invertebrates). Moreover, temperature-dependent clines (i.e. local adaptation) between populations represent diverging evolutionary histories that can elucidate how populations will respond to climate change 11,22,23.

However, recent evidence suggests that the rapid pace of climate change can disrupt local adaptation processes via shifting selection pressures 23.

**The ability to response to shifting selection pressure will depend on the genetic architecture and demographic processes found within different populations.**

* Need to consider both populations:
  + Physiological traits and underlying:
  + Genetic architecture
    - Important to consider genetic interactions between trait and direction of selection within multiple traits
  + Demographics; connectivity; genetic diversity

**Broad range species may not always have increased adaptive potential/Genetic architecture to overcome changes in selection pressures caused by climate change. Isolated populations across large ranges may all be affected, therefore entire species affected (see (Jump 2005)).**

* Thus, making it important to consider regional influences within species ranges…
* Long lived species can rapidly change allele frequencies within generation due to number of offspring produced

**Species regions (trailing/core/leading edge)**

**Apoly/Research objectives and aims**

# Methods

**# metrics that we measured in this manuscprit (metabolic rate, pha, hematocrit, enzymes, genetics)**

## Sampling

The tropical damselfish, *Acanthochromis polyacanthus* [citation: Bleeker 19xx], ranges from the southern Great Barrier Reef (GBR) to the central Philippines (mean depth: ; lat-lon). *A. polyacanthus* populations are thought to have propagated the Indo-Pacific during glacial times [when is glacial times] when lowered sea levels would have provided shallow water channels that acted as dispersal corridors24. However, such dispersal opportunities would have eliminated as water levels began to rise and reach present-day levels, as *A. polycanthus* do not possess a pelagic larval stage. *A. polyacanthus* perform parental care during embryonic and early life development, in socially monogamous pairs, where eggs are defended by both parents until fry are large enough to disperse into the surrounding habitat [citation: Planes -> Robertson 1973; Thresher 1983]. This unusual life history trait, among marine fish, coupled with *A. polyacanthus* inability to disperse between reefs separated by depths greater than 10-15m [citation needed], creates conditions that should promote local adaptation21; a broad geographic distribution across thermally variable environments, where gene flow is limited.

Adult *A. polyacanthus* were collected via professional collectors from June to December 2021 from six different reefs and two different regions (central GBR [Cairns] and southern GBR [Mackay]). Three reefs from locations around Cairns including, Tongue Reef ([-16.341, 145.773], *n =6*), Vlassof Cay ([-16.657, 145.990] *n =6*), and Sudbury Reef ([-16.996, 146.202] *n =9*), as well as from inshore islands and reefs in proximity to Mackay including: Cockermouth Island ([-20.772, 149.390] *n =8*), Keswick Island ([-20.908, 149.406] *n =4*), and Chauvel Reef ([southern; -20.863, 150.363] *n =5*; **Figure 1**). Cairns and Mackay collection regions are separated by XXX kilometers (spanning XX° in latitude).

Adult fish were held in separate 60L opaque aquariums ([DIMENSIONS]) inside an environmentally controlled aquarium room at the Marine and Aquaculture Research Facility at James Cook University (Townsville, Australia). Each aquarium contained a shelter (half a terra-cotta pot), constant aeration, and water flow (2 L/min) at set experimental conditions (see below). Fish were transferred to the experiment room that was used for trials on May 25th, 2022. Respirometry trials occurred from June 6th, 2022 – August 17th, 2022.

## Thermal conditions

Local thermal conditions for reefs within Cairns and Mackay locations were examined using temperature data collected via AIMS Temperature Logger data series for a subset of reefs (**ST1**) from each region (citation for AIMS data; **SF1**).

Experimental temperatures for metabolic and immunocompetence metrics were 27°C, 28.5°C, 30°C (IPCC 2050 projected temperatures citation), and 31.5°C (IPCC 2100 project temperatures under projection XXXXX citation). Once metabolic and immunocompetence trails were complete at a set treatment temperature, fish were exposure to +1.5°C increase at a rate of +0.5°C/day for three consecutive days. Fish were then given an additional five days to adjust to the new temperature treatment before the next sampling period began.

## Metabolic rate

Routine and maximum metabolic rate was measured via the rate of oxygen consumption using intermittent flow respirometry. Chambers were 1.5L in volume and custom built from PVC pipe and acrylic (**SF2**). The experimental setup consisted of two sumps (volumeL) with continuous water change and aeration, each containing four respirometry chambers submerged in water, placed in parallel, and out of view of other chambers (chambers were opaque except of lids). Each measurement cycle consisted of a fifteen second wait, three-minute-and-forty-five second measurement, and three-minute flush period. Each respirometry chamber unit included an independent brushless DC recirculation pump (flow rate 240L/H), associated vinyl tubing (composing ~1% of the total water volume), and oxygen sensor probes (multichannel FireStingO2, PyroScience GmbH, Aachen, Germany) with attached sensor spot material placed in-circuit. Oxygen sensor probes were calibrated to zero at the beginning of the experiment and/or if spot material was replaced and were calibrated to one hundred percent at the beginning of each trial. During flush periods an AQUAPRO (AP750LV) pumps (750L/H) was used to flush four chambers simultaneously. XXXX heaters and temperature sensors were used to ensure that experimental temperatures remained within +/-0.3°C of experimental temperature set points. UV filters, one-hundred-micron bag filters, and daily cleaning of equipment (bleach diluted to 200ppm with fresh water) ensured minimal background respiration rates. Fish were deprived of food 18-24 hours before trials began and sampled in a fully lit room to eliminate metabolic costs associated with digestion and photoperiod, respectively [citation].

### Maximum metabolic rate (MMR)

To measure maximum metabolic rate (MMR) fish were placed in a swim tunnel for ten minutes. During the first five-minute interval, the speed of the swim tunnel was slowly increased until fish displayed a change in gait swimming behavior, defined as a transitioning behavior between predominately pectoral swimming to predominately body/tail undulations (**SV1**). The speed of the swim tunnel that produced this intermediary transitional swimming behavior was maintained for the second five-minute interval to achieve maximum aerobic performance. Immediately after the ten-minute swimming period, fish were collected by hand and transferred to respiration chambers. Time between fish being placed in respiration chambers and the of data being recorded was measured to be less than ten seconds. MMR was measured over a thirty second interval via rolling regressions within the *‘*auto\_rate’ function included in the R package ‘*respR’* (v2.0.1). The steepest slope (highest oxygen consumption rate) with an *r2* threshold of 0.95 was used to determine MMR. MMR was measured prior to RMR.

### Routine metabolic rate (RMR)

Fish were randomly placed in individual respirometry chambers, and their oxygen consumption was measured over a 3.5 – 6hr ( =4.67hrs) period. Oxygen concentration (percent air saturation) was measured continuously every ~1.14 seconds and did not drop below 80% air saturation. Oxygen consumption rates were measured over a three-minute and forty-five second interval with an *r2* threshold of 0.95.Routine metabolic rate was measured by taking the mean of lowest five oxygen consumption slopes. Background respiration was measured at the start of each trial by measuring oxygen consumption within empty chambers for at least three consecutive cycles. Background respiration levels were minuscule and typically accounted for <2% of measured oxygen usage rates and were subsequently ignored. Mass of fish was measured at the end of respiratory trials, after fish had been euthanized and patted dry with paper towel to avoid the inclusion of excess moisture. The net respirometer volume of chambers ranged from 1:116 to 1:36 depending on the size of each fish. Oxygen consumption rates were converted from percent air saturation values via the *‘convert\_rate’* function within the R package *respR* [citation].

## Phytohemagglutinin (PHA)

PHA injections produce a cell-mediated response (e.g., inflammation and T-cell proliferation), representing a local cellular immune response25. Fish were injected in the caudal peduncle with 0.3 mL of phytohemagglutinin (PHA; L8754 Sigma-Aldrich, 45 ug 10 uL-1). Immune responses (i.e., immunocompetence) of fish was determined by measuring the swelling of the injected area with XXX pressure sensitive calipers pre-injection as well as ~18-24 hours post-injection.

## Maximal enzyme activity analysis

White muscle tissue (dissected from muscle between the dorsal fin and lateral line) was used to examine the maximal enzyme activity of lactate dehydrogenase (LDH) and citrate synthase (CS). Temperatures including 10°C, 20°C, 30°C, 40°C, and 50°C were used to determine maximal enzyme activity and the associated thermal performance curve. White muscle tissue was used for the maximal enzyme activity analysis because it plays an important role in locomotion activities, compromises most of the body mass for *A. polyacanthus*, and is easily accessible (more information on why w. muscle tissue was used; citation). White tissue samples were extracted from fish immediately after fish had been euthanized, placed in liquid nitrogen, and then transferred to a -80°C freezer for long term storage.

The maximal enzyme activity analysis used was adapted from previous studies26,27 Seebacher (2003), McClelland (2005). Samples from *n =38* fish were defrosted on ice. A sterile scalpel blade was used to extract a tissue sample (20 – 40 mg). Extracted tissue samples were homogenized via a microtube homogenizer (BeadBug 6, Benchmark Scientific, Edison NJ – double check) in 1:10 proportions with a buffer consisting of 50 mmol L-1 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mmol L-1 ethylenediaminetetraacetic acid (EDTA), 0.01% Triton X-100, and 99.99% Milli-Q water, and adjusted to pH 7.4 with sodium hydroxide (NaOH). A subset of homogenized tissue was extracted for LDH, and CS. Homogenized tissue samples used for the LDH assay were centrifuged (Eppendorf Centrifuge 5430, Hamburg, Germany) at 150 rpm for <3 seconds. Homogenized tissue samples used for the CS assay were not centrifuged to allow mitochondria to be retained within the supernatant.

Absorbance readings were measured with a spectrophotometer (2-s readings, with 20 readings over 13 minutes: UV5, Mettler-Toledo, Columbus, OH). Treatment temperatures were maintained with a Loop L100 circulation thermostat (Lauda, Lauda-Königshofen, Germany). All samples were measured in triplicate and included a blank sample.

LDH was assayed in 0.5 mmol L-1 of β*-*nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH)-Tris solution (pH 7.4). and 50 mmol L-1 of sodium-pyruvate-NADH-Tris solution (pH 7.4). NADH absorbance was measured at a wavelength of 340 nm (Seebacher 2003). CS was assay in 2 mmol L-1 5,5’-dithobis-(2-nitronemzoic acid) (DTNB)-ethanol solution, 12 mmol L-1 acetyl coenzyme A-lithium salt-Milli-Q solution, and 50 mmol L-1 oxaloacetic acid-Tris solution (pH 8.0). DTNB absorbance was measured at a wavelength of 412 nm (Seebacher 2003; Blank 2004).

The mean of the three absorbance slopes was used to determine maximal enzyme activity. Background activity was subtracted from sample absorbance slopes (citation). Final maximal enzyme activity levels were calculated in units per milligram tissue (U mg-1 tissue) using the following formula:

[ENTER FORMULA HERE}

(Description of variables in formula).

## Hematocrit

Blood used from hematocrit samples was collected at the end of the experiment when the temperature treatment was 31.5°C, XX days after the final PHA immunocompetence trial had been completed. Microcapillary tubes were used to collect XX ul of blood from the caudal vein. Collected blood samples were centrifuged at XXX rpm for XX seconds to separate red cells from blood plasma. The proportion of blood volume occupied by red blood cells (hematocrit) was recorded by usinga ruler to first measure the space of the microcapillary tube that was occupied by the total blood volume (packed red blood cells and blood plasma), followed by measuring the space occupied by just packed red blood cells. Hematocrit scores were calculated using the following formula:

## Statistical analysis

Generalized linear mixed effect models were used to model metabolic, immunocompetence, enzyme activity, and hematocrit responses within Cairns and Mackay region fish to temperature treatments. All metabolic models were run using a gaussian distribution, unless otherwise stated. To model metabolic responses including NAS, RMR, and MMR, independent variables including, region and temperature were modelled as an interaction and fish mass (centered) was used as a covariate. Individual identification codes for each fish were used as a random factor due to repeated measures. The model for resting metabolic rate included the additional covariate of testing runtime. The same variables included in the GLMM used for metabolic rates, were used for modelling PHA immunocompetence response and enzyme (lactate dehydrogenase) activity. However, for the PHA swelling response model instead of a gaussian distribution, a gamma distribution was used with an inverse link. For the enzyme analysis for lactate dehydrogenase model tissue mass (centered) was used instead of fish mass. To model the (combined region) correlation between lactate dehydrogenase activity and temperature, temperature was modelled as a continuous numerical variable and third order polynomial, tissue mass (centered; fixed), and individual fish identification codes as a random factor. Hematocrit was modelled as a linear regression with percent packed blood cells as the dependent factor and region as an independent variable.

All statistical analysis was conducted in R (v 4.2.2). GLMMs were run using the ‘glmmTMB’ function within the ‘*glmmTMB’* (v.1.1.5). Model selection occurred using the function ‘AICc’ via the *‘MuMin’* (v.1.47.1). Visual and statistical performance of models was checked via both the ‘check\_model’ function in the *‘performance’* (v. 0.10.0) package and the ‘simulateRedisuals’ and ‘testResiduals’ functions in the ‘*DHARMa’* (v. 0.4.6) package. The *‘emmeans’* (v. 1.8.2) package was used to extract estimated marginal means from models that were used to tested for statistical significance. All figures were made using the ‘*ggplot2*’ (v. 3.4.0) package.

# Results

In total XX fish were sampled over the duration of the experiment (**Table 1**). Resting metabolic rate, maximum metabolic rate, aerobic scope, immunocompetence, maximal enzyme analysis, hematocrit samples, and genetic sequencing data were all collected for *n =38* fish in total, sampled from Tongue Reef (*n =6*), Sudbury Reef (*n =9*), Vlassof Cay (*n =6*), Cockermouth Island (*n =8*), Keswick Island (*n =4*), and Chauvel Reef (*n =5*). Additional samples were included for the respirometry and immunocompetence trials, however, not all fish survived the duration of the experiment.

## Metabolic rate

### Net aerobic scope

Significant differences in net aerobic scope (NAS) were seen between fish from Cairns and Mackay regions of the GBR at warmer temperatures (**Figure2a**). At 30°C (*p =0.0050,* [CI: 0.732, 4.06]) and 31.5°C (*p =0.0094,* [CI: 0.565, 3.99]) fish from the Cairns region had significantly larger aerobic scope capacity than fish from Mackay. The net aerobic capacity of Cairns region fish was 2.40 and 2.28 MgO2\_hr of oxygen larger at 30°C and 31.5°C, respectively, both representing an increase in MgO2\_hr of 30% over Mackay region fish. No significant differences were identified between Cairns and Mackay region fish at 27°C (*p = 0.70*), or 28.5°C (*p = 0.086*). Changes in NAS across tested experimental temperatures occurred across greater increments (steeper trends) within fish from the Cairns region compared to fish from Mackay region. NAS for Cairns region fish was lowest at 27°C (8.04 MgO2 hr-1), and highest at 30°C (10.40 MgO2 hr-1), an increase of 29%. Interestingly, at 28.5°C and 31.5°C Cairns region fish showed similar NAS values of 9.72 MgO2 hr-1 and 9.80 MgO2 hr-1, respectively. Differences between NAS values within Mackay region fish were much less pronounced. The lowest NAS value for Mackay region fish was at 31.5 °C (7.52 MgO2 hr-1), while the highest value at 27 °C (8.37 MgO2 hr-1), just 0.85 MgO2 hr-1 or 11% higher.

### Maximum metabolic rate

Significant differences in maximum metabolic rate between Cairns and Mackay region fish were seen at similar temperatures as NAS. At 30°C (*p =*0.0052, [CI: 0.697, – 3.92]) and 31.5°C (*p =*0.0017, [CI: 1.02, 4.33]) Cairns region fish had significantly higher maximum metabolic rates than fish from Mackay (**Figure2b**). At 30°C and 31.5°C Cairns region fish maximum metabolic rate was 16% (2.31 MgO2 hr-1) and 19% (2.68 MgO2 hr-1) higher than Mackay region fish, respectively. No significant differences were identified between Cairns and Mackay regional fish at cooler temperatures including 27°C (*p* =0.82) and 28.5°C (*p* =0.079). When comparing within region performances, Cairns region fish displayed an increase of 10%, 16%, and 19%, at 28.5°C, 30°C, and 31.5°C, respectively, compared to MMR at 27°C. However, among Mackay regional fish little difference was seen in MMR between temperatures. Compared to 27°C increases in MMR at 28.5°C, 30°C, and 31.5°C were 2%, 1%, and 1%, respectively for Mackay region fish.

### Resting metabolic rate

No significant differences were seen in RMR when comparing fish from Cairns and Mackay regions at tested experimental temperatures (**Figure2c**), however, RMR displayed a positive relationship with temperature. Among Cairns region fish, RMR was significantly higher at 31.5°C compared to RMR at 27°C (*p* <0.0001, [CI: -2.07, -0.66]), 28.5°C (*p* <0.0001, [CI: -1.99, -0.65]), and 30°C (*p* =0.0077, [CI: -1.50, -0.17]). From the lowest temperature, 27°C, within Cairns region fish RMR increase by 1%, 9%, and 24% at 28.5°C, 30°C, and 31.5°C, respectively. Among Mackay region fish RMR had the greatest increase between 28.5°C and 30°C; RMR at 30°C (*p27.0–30.0* <0.0022, [CI*27.0–30.0*: -1.78, -0.29]; *p28.5–30.0* <0.035, [CI*28.5–30.0*: -1.53, -0.039]) and 31.5°C (*p27-31.5* <0.0001, [CI*27–31.5*: -2.17, -0.66]; *p28.5–31.5* =0.0006, [CI*28.5–31.5*: -1.91, -0.40]) were significantly different than RMR at 27°C and 28.5°C. From 27°C RMR of Mackay region fish increased by 5%, 19%, and 26% at 28.5°C, 30°C, and 31.5°C, respectively.

## Phytohemagglutinin (PHA)

No significant differences were found in PHA immune swelling response between Cairns and Mackay region fish at any of the tested treatment temperatures. For fish from both regions PHA swelling response was highest at 28.5°C and lowest at 31.5°C. Temperatures of 27°C and 30°C produced intermediary PHA swelling responses.

PHA swelling response was significantly lower at 31.5°C compared to swelling responses at 28.5°C (*pCairns* =0.0006, [CICairns: 0.086, 0.41]; *pMackay* =0.0006, [CIMackay: 0.086, 0.41]) and 27°C (*pCairn* =0.0016, [CICairns: 0.057, 0.32]; *pMackay* =0.046, [CIMackay: 0.0013, 0.23]) in fish from both Cairns and Mackay regions (**Figure 3**). Within fish from the Cairns region at 31.5°C swelling response was just 31% and 36% of the swelling response seen at 28.5°C and 27°C, respectively. The swelling response for fish from Mackay at 31.5°C was 21% and 43.5% of that seen at 28.5°C and 27°C, respectively.

## Enzyme analysis

### Lactate dehydrogenase

Lactate dehydrogenase activity was positively correlated with temperature (*p* <0.0001, [CI: 1.81, 2.64], *R2 =*0.79), however, no significant differences were seen in LDH activity between Cairns and Mackay region populations at any of the tested experimental temperatures for lactate dehydrogenase activity: 20°C (*p* =0.14), 30°C (*p* =0.22), 40°C (*p* =0.064), and 50°C (*p* =0.28) (**Figure 4**).

## Hematocrit

No significant difference was observed in hematocrit levels between Cairns and Mackay region fish (*p* =0.058). Packed red blood cells composed 22.4% and 25.9% of whole blood for Cairns and Mackay region fish, respectively.

## Genetic sequencing

# Discussion

**Details pertaining to respiration trials are provided below following the guidelines outlined by Killen *etl al.* (2021)**28**.**

## Sequencing and genomic analyses:

The novel approach of shallow whole genome sequencing (sWGS) will allow genome-wide associations with phenotypic traits to be detected. Reduced genetic diversity in areas of the genome where an allele is under selection, distinct patterns of haplotype structure, and linkage disequilibrium will be explored to determine the genetic changes that underpin local adaptation within different regions. The phenotype-genomic integrated approach will allow for an increased understanding of the evolutionary events that occurred surrounding local adaptation within a widespread species.

#### Chauvel Reef (southern)

Despite sharing a similar latitude Chauvel Reef (southern) represents in an inshore reef, rather than an inshore island as represented by Cockermouth and Keswick Island. When analyzed separately from Cockermouth and Keswick Island, Chauvel Reef showed a number of significant differences

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