# 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

**# Is it necessary to mention the common garden experiment design? since it is not relevant for the**

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

## Study species and

The tropical damselfish *Acanthochromis polyacanthus* was used in the present study because its life history traits are well suited to promote local adaptation21. Specifically, *A. polyacanthus* possesses direct development (ref) and both parents provide care during embryonic and early juvenile development (ref). This species is distributed broadly across the Indo-Pacific (depth: , lat and long of range) and this geographic distribution encompasses a range of thermal environments (). Research to date indicates that fish are unable to easily disperse across large distances or depths (<10-15m: citation needed).

Adult *A. polyacanthus* were collected via professional collectors from June to December 2021 from six different reefs and two different regions (Cairns and Mackay). Three reefs from locations around Cairns including, Tongue Reef (-16.341, 145.773), Vlassof Cay (-16.657, 145.990), and Sudbury Reef (-16.996, 146.202), as well as from inshore islands and reefs in proximity to Mackay including: Cockermouth Island (-20.772, 149.398), Keswick Island (-20.908, 149.406), and Chauvel Reef (southern; -20.863, 150.363; **Figure 1**). Cairns and Mackay collection regions are separated by a distance of XXX kilometers (spanning XX° in latitude).

Adult fish were held in separate 60 L 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 (~X mL/min) at set experimental conditions (see below). Fish were transferred to the aquarium room that was used for the experiment on May 25th, 2022. All trials outlined below occurred from June 6th to August 17th, 2022. All research was conducted under Animal Ethics Approval XXXX.

## Thermal conditions

To understand local thermal conditions for reefs within the Cairns and Mackay locations, temperature data for a subset of reefs from each region were collected via AIMS Temperature Logger data series (citation for AIMS data; **SF1**), using temperature loggers at a depth between 10-15 m. Average summer temperatures (Dec-Mar? – double check) were determined to be ~27.0°C and ~28.5°C, for reefs around Mackay and Cairns, respectively (**SF 1**).

Experimental temperatures for the repeated aerobic physiology and immune response testing were 27°C, 28.5°C, 30°C and 31.5°C. These temperatures are within the natural range of both populations (27 and 28.5 °C) or temperatures that occur infrequently a maximal temperature or with marine heat waves (30 and 31.5°C), as well as being projected to occur regularly by the end of the century (IPCC 2100 project temperatures under projection XXXXX citation). Testing began at the coolest temperature of 27°C, and once aerobic physiology and immune response testing was complete, fish were warmed to the next temperature of +1.5°C 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. This process was repeated for all testing temperaures.

## Aerobic physiology

Routine and maximum metabolic rate was determined via measuring the rate of oxygen consumption using intermittent flow respirometry. Chambers were 1.5 L in volume and custom built from PVC pipe and acrylic (**SF2**). The experimental setup consisted of two sumps (volume L) with continuous water exchange and aeration, each containing four respirometry chambers submerged for temperature. Each chamber was placed in parallel and fish could not view each other (i.e., chambers were opaque except of lids). Each measurement cycle consisted of a 15 s wait, 3:45 s measurement, and 3 min flush period. Each respirometry chamber unit included an independent external brushless DC recirculation pump (brand or model, flow rate 240 L h-1 ), associated vinyl tubing (composing ~1% of the total water volume), and an in-circuit oxygen sensor probe with attached sensor spot material (multichannel FireStingO2, PyroScience GmbH, Aachen, Germany). Oxygen sensor probes were calibrated to 0% at the beginning of the experiment and if spot material was replaced, and were calibrated to 100% percent at the beginning of each trial. During flush periods a pump (AQUAPRO, AP750LV, 750 L h-1) was used to flush each set of four chambers simultaneously. XXXX watt heaters and temperature sensors were used to ensure that temperatures remained within +/-0.3°C of the desired experimental temperature set points. Minimal background respiration was achieved through UV filtration, particle filtration (100 µm bag filters), and daily cleaning of equipment (bleach diluted to 200 ppm with fresh water). Fish were deprived of food for 18-24 h before aerobic physiology trials began and trials were conducted in a fully lit room to eliminate metabolic costs associated with digestion and photoperiod, respectively [citation].

To measure maximum oxygen consumption as a proxy for maximum metabolic rate (MO2Max) fish were placed in a swim tunnel for 10 min. During the first 5 min interval, the speed of the swim tunnel was slowly increased until fish displayed a change in gait swimming behavior, defined as a transitioning 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 another 5 min interval to achieve maximum aerobic performance. Immediately after the 10 min swimming period, fish were collected by hand and transferred to respiration chambers. The time between fish being placed in respiration chambers and the start of data being recorded was measured to be less than 10 s. Oxygen concentration (percent air saturation) was measured continuously every ~1.14 s. MO2Max was measured over a 30 s 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 MO2Max. MO2Maxwas measured prior to routine metabolic rate (MO2Routine).

Fish were randomly placed in individual respirometry chambers, and their oxygen consumption was measured over a 3.5 – 6 hr ( = 4.67hrs) period. Oxygen concentration was again measured continuously every ~1.14 seconds and did not drop below 80% air saturation. Oxygen consumption rates were measured over a 3:45 s interval with an *r2* threshold of 0.95.MO2Routine was measured by taking the mean of the 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. The mass of fish was measured at the end of all respirometry 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 L 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].

Absolute aerobic scope (AS) was calculated from…

## Immune response

To test the sensitivity of the immune system an immune challenge was undertaken. This involved injecting phytohemagglutinin (PHA) to produce a cell-mediated response (e.g., inflammation and T-cell proliferation), representing a local cellular immune response24. Fish were injected in the caudal peduncle with 0.03 mL of PHA (PHA; L8754 Sigma-Aldrich, 45 ug 10 uL-1) dissolved in phosphate buffered saline (PBS), made to a ratio of 1 mg PHA to 1 mL PBS. The immunocompetence of fish was determined by measuring the injection area pre-injection with pressure sensitive calipers (model, XXX) as well as ~18-24 hours post-injection and determining the change (i.e., post-pre).

## Fish sampling

At 31.5 … end experiment… Took blood… measured… took tissues…

## 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 immune challenge 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 using a 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:

## Enzyme activity

White muscle tissue dissected from between the dorsal fin and lateral line was used to examine the maximal enzyme activity of lactate dehydrogenase (LDH) and citrate synthase (CS). Testing temperatures of 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 storage.

The maximal enzyme activity methods used here was adapted from previous studies25,26 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 s. 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 every 2 s, with 20 readings over 13 min (UV5, Mettler-Toledo, Columbus, OH). Testing temperatures were maintained with a Loop L100 circulation thermostat (Lauda, Lauda-Königshofen, Germany). All samples were measured in triplicate and included a blank control.

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).

## Statistical analysis

Generalized linear mixed effect models were used to test the aerobic metabolic physiology, immune response, hematocrit and enzyme activity responses within Cairns and Mackay region fish to temperature. All aerobic metabolic models were run using a gaussian distribution, unless otherwise stated. To model metabolic responses including MO2Routine, MO2Max, and AS, independent variables including, region and temperature were modelled as fixed factors with 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 the repeated measures design. The model for MO2Routine included the additional covariate of testing runtime. The same fixed variables of region and temperature were included in the modelling PHA immunocompetence response and enzyme (LDH) 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.

## Genetic sequencing

# 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

### MO2Rest displayed a positive relationship with temperature, but nthe pattern of increasing MO2Rest**a; Stats**. For both populations, MO2Rest was similar between the lowest two testing temperatures from X to Y mgO2hr-1 (not sig post-hoc). Additionally, MO2Rest was significantly higher at both 30 and 31.5C than at 27 and 28.5C (stats from overall post-hoc of model). While not significant, Cairns fish exhibited the greatest increase in MO2Rest from 30-31.5C of 15%, while Mackay fish MO2Rest increased by 14% between 28.5 to 30C and by 7% from 30 to 31.5

The relationship between MO2Max and temperature differed between Cairns and Mackay region fish (stats anova of model; Figure 2b). Fish from Mackay showed a flat response where MO2Max was similar of XXX mgO2hr-1 on average at all temperatures. While for Cairns region fish an increase in MO2Max was found across temperatures (27-28.5C: 10%, 28.5-30C: X%, and 30-31.5CY%). This difference resulte din Cairns region fish having significantly higher MO2Max at 30°C by 16% (2.31 MgO2 hr-1; *p =*0.0052, [CI: 0.697, – 3.92]) and 31.5°C by 19% (2.68 MgO2 hr-1 ; *p =*0.0017, [CI: 1.02, 4.33])(**Figure2b**).

Similarly, sdepending on with AS peaking at 27-28.5 for Mackay fish (9.72 MgO2 hr-1) and around 30C for Cairns fish(10.40 MgO2 hr-1)**c; stats for temp factor**This resulted in significant differences between the two regions ais enhancedASby ed

Immune response

## 

The immune swelling response exhibited a curved response with a peak at 28.5C (stats on temp; Figure X), but no differences were found between Cairns and Mackay region fish at any of the tested temperatures (stats). For fish from both regions the immune response was lowest at 31.5°C (26?% and 40?% reduction; sig from who? 27 and 28.5C), and intermediate at temperatures of 27°C and 30°C (NSig from who?).

at 31.5C

## 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**).

## Genetic sequencing

# Discussion

**Details pertaining to respiration trials are provided below following the guidelines outlined by Killen *etl al.* (2021)**27**.**

## 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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