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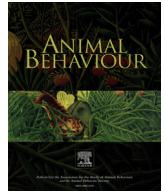
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Rise and fall: increasing temperatures have nonlinear effects on aggression in a tropical fish

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Environmental conditions are fundamental drivers of animal behaviour. Aggression in ectotherms is a marked example and is often considered positively correlated with temperature. However, many studies exploring this relationship, and its important consequences, focus on a few temperatures and implicitly assume a linear relationship between the two variables. This may limit our understanding of the influence of temperature on aggression. We measured levels of aggression in *Neolamprologus pulcher*, a cichlid fish commonly used as a model in studies of aggression-based social hierarchy, across a range of temperatures in a mirror aggression test. We used temperatures expected from their natural range with a 2 °C extension on either end. As predicted by thermal performance curves, rates of aggression increased with temperature at the lower end of the temperature range. After reaching a peak, any further increase in temperature resulted in a considerable drop in rates of aggression. Exploring responses at a finer temporal scale, we found that the influence of high temperatures changed dramatically during the 25 min trials. Early in the trials the frequency of aggression increased linearly with temperature, across all temperatures. The initially high levels of aggression at the higher temperatures were not sustained and dropped with increasing duration of exposure to the mirror at high temperatures. Together these findings provide a more comprehensive understanding of the influence of short-term exposure to different temperatures on aggression, such as acute exposure to increased temperatures caused by global warming and associated rapid thermal fluctuations, in African rift lake fishes and in ectotherms more generally. Moreover, our results highlight the importance of measuring aggression across a range of temperatures or otherwise account for nonlinear changes in thermal performance.

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The observed and expected temperature fluctuations are one of the most concerning sources of human-induced environmental change. Temperature changes impact the physiology and behaviour of all animals, with especially profound, direct effects on poikilotherms like aquatic ectotherms (Abram et al., 2017; Crowley & Pietruszka, 1983; Ratnasabapathi et al., 1992). These effects can be complex, with multiple underlying physiological processes involved (Schulte et al., 2011). While the general influence of temperature on behaviour has received substantial research attention, a more refined understanding of the impacts of temperature change requires exploring the nuances (Franklin & Hoppeler, 2021; Lefevre et al., 2021).

Agonistic interactions play a crucial role in maintaining many animals' social systems. Aggressive displays and/or physical conflict are a core behavioural mechanism through which social interactions with both heterospecifics and conspecifics are modulated (Frommen, 2020; Huntingford & Turner, 1987; Ward & Webster, 2016). The formation and stability of dominance hierarchies, for example, is a dynamic process involving changes in the frequency, type and intensity of aggressive behaviours (Holekamp & Strauss, 2016). In ectotherms, and poikilotherms more generally, levels of aggression often increase with temperature (Bissell & Cechala, 2019; Crowley & Pietruszka, 1983; Kua et al., 2020). Exposure to more rapid fluctuations and more extreme temperatures are increasingly likely under climate change (Vasseur et al., 2014), and predicted to be especially impactful in tropical ecosystems where animals have evolved to stable thermal regimes (Deutsch et al., 2008; Morgan et al., 2022). Still, there is limited understanding of how changes in aggression will be affected and what the

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consequences of such changes will be. For example, temperature-induced changes in aggression may upset social dynamics and raise questions around when or why dominance hierarchies may change, depending on whether dominance rank is supported by absolute or relative levels of aggression (Strauss & Shizuka, 2022).

Studies exploring the relationship between temperature and aggression often tend to use a limited selection of specific temperatures. Indeed, several studies on fishes have compared aggression with just two (Barki et al., 2022; da Silva-Pinto et al., 2020; Kua et al., 2020; Kvarnemo, 1998; Lopez et al., 2018; Matthews & Wong, 2015; Ratnasabapathi et al., 1992) or three (Brandão et al., 2018; Carmona-Catot et al., 2013) temperatures. Hence, there is a lack of understanding of the nuanced effects of temperature on aggression, specifically how aggression changes across the thermal range of ectotherms, which experience more profound effects of temperature on their behaviour (Paaijmans et al., 2013). The link between aggression and temperature should be expected to follow physiological responses in ectotherms. Such physiological performances tend to follow thermal performance curves, with lower performance at low temperatures, increasing with temperature up to a maximum and then a drop with further increases (Jutfelt, 2020; Schulte et al., 2011). Such effects have been demonstrated for example in guppies, *Poecilia reticulata*, which show an initial increase and then a decrease in levels of activity across the range of temperatures within their natural range (Wilson, 2005). Furthering our understanding of the relationship between aggression and temperature across a wider thermal range is therefore important to better inform our predictions of the effects of climate change on aggressive behaviour, and the secondary consequences on the maintenance of stability of complex social systems.

In this study we explored the effect of temperature on aggression in a Lake Tanganyikan cichlid fish, the Princess of Zambia, *Neolamprologus pulcher*, a model species commonly used to understand aggression and its role in social dynamics (Reddon et al., 2012; Riebli et al., 2011; Zöttl et al., 2013). These fish are obligate cooperative breeders that live in social groups up to 20 individuals throughout their life (Groenewoud et al., 2016; Jungwirth et al., 2023). Group hierarchies are maintained via aggressive and submissive interactions, especially between similar-sized individuals (Ligocki et al., 2015), and aggression is crucial for defence against hetero-specific competitors and predators (Groenewoud et al., 2016). During agonistic encounters both sexes use a broad range of visual displays to show their motivation and resource-holding potential in restrained aggression (Balzarini et al., 2014, 2017). If agonistic encounters are not solved by such displays they readily escalate to overt attacks with physical contact, including bites (Josi & Frommen, 2021). Such overt attacks have been demonstrated to be energetically costly compared to restrained displays, both in *N. pulcher* (Grantner & Taborsky, 1998) and in other cichlid species (Neat et al., 1998; Ros et al., 2006). *Neolamprologus pulcher* is a bottom-dweller that defends year-long territories of up to 1 m in diameter around shelters on sandy to rocky substrate (Freudiger et al., 2021). These territories cluster together in colonies (Groenewoud et al., 2016) and can be found in shallow coastal waters in depth from 2 m to below 40 m (Konings, 2019). At these depths, tropical lakes, including Lake Tanganyika, face temperature fluctuations and are known to be affected by global warming (Kraemer et al., 2015; O'Reilly et al., 2003; Verburg et al., 2003). The temperature ranges within these depths in the lake vary between 23 and 29 °C across seasons, but with mean temperatures increasing, and predicted to continue increasing, by 0.225 ± 0.112 °C per decade (Kraemer et al., 2015; Phiri et al., 2023). While sexually mature *N. pulcher* individuals will move into the water column to feed on plankton for a certain amount of time, they do not appear to use behavioural thermoregulation routinely as they

are closely tied to their territories throughout their life (Bruintjes et al., 2010; Jungwirth et al., 2023).

We used mirrors to induce aggression across a range of temperatures. The behaviour of animals towards their mirror image ('mirror test') is routinely used to measure aggression levels in many species including mammals (Haigh et al., 2017; Svendsen & Armitage, 1973), birds (Branch et al., 2015) and fishes (Adriaenssens & Johnsson, 2013; Lissmann, 1932; Meliska et al., 1980; Rowland, 1999). While the ecological relevance of mirror aggression tests in some fishes is disputed (Balzarini et al., 2014; Oliveira et al., 2005), and can depend on the type of mirror used (Li et al., 2018), they have been shown to elicit ecologically meaningful results in *N. pulcher*, both in the laboratory (Balzarini et al., 2014) and in the field (Josi & Frommen, 2021), and are used widely for exploring aggression in this species (Heg et al., 2004; Hotta et al., 2018; Reddon et al., 2012; Riebli et al., 2011; Spinks et al., 2017). In the present study we specifically aimed to explore: (1) whether aggression changes with temperature across a typical range as expected from thermal performance models of physiology; (2) whether the type of aggression changes with temperature, specifically, whether overt, more (physiologically) costly aggression and restrained (less costly) display behaviour both change in similar patterns; and (3) the effect of temperature on how long aggression lasts within a trial, specifically, whether the frequency of overt displays across trial time changes with temperature. We predicted that at higher temperatures the frequency of aggressive behaviours will drop more quickly, as physiological costs accumulate over a trial faster in warmer conditions.

METHODS

Experimental Animals and Holding Conditions

We used laboratory-bred first-generation offspring *N. pulcher*. The parental generation was caught at the southern tip of Lake Tanganyika, close to the village of Chikonde, Republic of Zambia (Freudiger et al., 2021). Prior to testing, fish were kept in mixed-sex aggregations of two to eight each in 220-litre tanks (180 × 35 cm and 35 cm deep), which could be subdivided into smaller compartments, depending on group size. Tanks were aerated and filtered by internal sponge filters. Pale-coloured, small-grain, coral-mixed gravel was used as a substrate. Shelter caves were provided in the form of large clay pots. The water temperature was controlled at the room level. One month prior to experiments temperatures were stabilized at 26 ± 1 °C. In the year prior to this the fish had experienced temperatures between 23 and 30 °C, as the fish room allowed for variation within this range. The room was maintained on a 12:12 h light:dark cycle and a 30 min sunset and sundown phase of gradual lighting programmed with Fluval Aquasky LED lights. All fish were fed to excess 5–6 days per week, with diet alternating between flake food (JBL Tanganyika) and thawed frozen invertebrates (bloodworms, brine shrimp, *Daphnia*, copepods). During the experiment, all fish were fed after the last trial of the day. The eight fish used in the experiment were randomly selected from the pool of adult fish. They originated from different stock tanks and were not related to each other. They had not formed pair bonds and had not mated for at least 6 months.

Experimental Set-up

The mirror tests were conducted in two 180 × 35 cm and 35 cm high tanks, which were divided into six equal-sized chambers using opaque plastic partitions. A temperature gradient was established from one end of the tank to the other (Table 1), using a slightly modified version of previously used thermal gradient tanks

Table 1
Temperature (°C) of chambers used in the testing tank

Chamber	Mean	SD
1	31.6	0.4
2	30.4	0.3
3	28.8	0.4
4	26.6	0.2
5	24.9	0.3
6	23.0	0.3
7	21.8	0.4
8	21.3	0.4

(Boltaña et al., 2013; Jones et al., 2019). To that aim, one end of the tank was heated with two 50 W immersion heaters. Clear Plexiglas lids were installed 10 cm above the water surface to reduce the loss of heat from the surface of the water. The temperatures were based on a recent study of aggression in a cichlid from Lake Tanganyika (Kua et al., 2020), and on current and projected surface temperatures, where summer temperatures have regularly reached over 29 °C in waters up to the first 40 m depth (Plisnier et al., 2018) and have been increasing and are expected to continue to increase (Kraemer et al., 2015; Tierney et al., 2010). The tanks were filled to a height of 25 cm with dechlorinated tap water and the bottom was covered with 1.5 cm of fine pale sand. Each chamber was furnished with an internal sponge filter for aeration, and half a flowerpot as a refuge at one side of the chamber, with the opening oriented towards the other side. Guiding rails were installed next to the divider opposite the shelter which allowed us to insert and remove a mirror with minimal disturbance (Balzarini et al., 2014).

Experimental Procedure

We used a repeated measures design with all fish tested in 10 different temperature chambers, with each fish tested in each chamber twice. Subjects were introduced to the experimental compartments in the afternoon (1500–1600 hours) before the mirror trials taking place the following day, thus allowing them to acclimatize overnight for at least 16 h. Before the first day of testing, standard length was measured to the nearest mm using a measuring board. The first mirror test trials were started from 0900 each day. Two trials using the same chamber were conducted per fish per day, with at least 4 h between trials. Subjects were tested in a different order each day. Fish were pseudorandomly allocated to a different chamber each day so that there was no sequential order of exposure to different temperatures and fish were not moved between chambers that differed by more than 5 degrees. After moving the fish, we monitored them for at least 15 min to be able to react to any potential behavioural changes indicating a lack of wellbeing. Fish only showed minimal signs of stress and recovered quickly after being moved.

Table 2
Ethogram of aggressive behaviours scored for each mirror trial

Aggression type	Behaviour	Description
Overt	Biting	Biting attempt, where the snout makes physical contact with the mirror and/or the mouth is opened and closed
	Ramming	Fast approach ending in physical contact with the mirror, mouth remains closed
Restrained	Fast approach	Swimming at high speed towards the mirror, opercula spread. Stopping before making contact with the mirror
	Fin raise	All fins are maximally spread; fish is close to the mirror (1.5 body lengths), either facing it or showing its side ¹
	Opercula spread	Opercula spread when facing the opponent, but not combined with a fast approach
	Head down	Body inclined downwards, up to 60°. Unpaired and pelvic fins are spread, fish 'trembles'
	Parallel swimming	Swimming repeatedly back and forth along mirror, body parallel to mirror, at close distance
	Frontal facing	Fish approaches and faces the mirror head on within 2 cm of the mirror; holds position for more than 1 s but does not make physical contact ²

¹ If the fish moved more than 45 degrees in vertical or horizontal axis while displaying the fins continuously, we counted it as a second display.

² Based on 'watching' behaviour as per Kohda et al. (2015).

Temperatures of each chamber were taken at 0800 and 1600, and again immediately after each trial allowing us to capture fine-scale measures of temperature for each trial. This was to account for the impact even small differences in temperature can have on behaviour, as per activity in reef fish (Biro et al., 2010).

Trials

The mirror was inserted into the chamber opposite the flow-erpot refuge immediately before the start of the trial. The behaviour of the fish was then captured using a video camera for 25 min, after which the trial ended, and the mirror was removed from the chamber.

Behavioural Measurements

For each trial, aggressive behaviours were scored using the BORIS software (Friard & Gamba, 2016). The scorer (N.R.J.) was blind to temperature in each trial, as temperature data were kept separate. Note, however, that in some cases it would have been possible to guess the temperature chamber and approximate temperature when the edge of the tank was visible for a fish tested in an end chamber. Behaviours were defined as either overt or restrained aggression as per a modified ethogram developed by Balzarini et al. (2014; see Table 2). All aggressive behaviours were counted as events. For analyses, aggression measures were summed into an overt and restrained category, respectively (Balzarini et al., 2014; Josi & Frommen, 2021). Technical problems occurred during two trials, and these were discarded, so that in total data from 158 trials were analysed, for eight fish across the range of trial temperatures.

Statistical Analyses

Analyses were conducted using R 4.2.2 (R Core Team, 2022). To account for the expected nonlinear influence of temperature on levels of aggression we fitted hierarchical general additive models (GAM), with the 'mgcv' package, using restricted maximum likelihood (REML) and a Poisson link function for counts of aggression, or the identity link function for models fitting scaled aggression. GAM models estimate the effective degree of smoothness (edf) of the model terms as part of the fitting process (Wood, 2011, 2018) but also allow the incorporation of nested or other random factor structures similar to mixed-effect models (Pedersen et al., 2019). The basis dimensions (k), which control the number of basis functions and sets the maximum degrees of freedom for smooth terms in the model, and the model fit were evaluated using the gam.check() function as per Lombardo et al. (2020). We plotted the fitted models using the 'tidygam' package (Coretta, 2023), which

returns model predictions from GAM models, and allows for the exclusion of model terms in those predictions in order to plot the predicted effects of specific terms in the fitted model.

Given that we used a repeated measures design, testing each fish in 10 temperatures, with two trials per temperature, we established the level of repeatability within fish, that is, whether differences in aggression were consistent across temperatures and days of testing. To this end we calculated repeatability 'R' using the 'rptR' package in R. This package builds on the functions developed for mixed-model analysis with the addition of parametric bootstrapping to provide reliable estimates for 'R' and the uncertainty surrounding these estimates (Stoffel et al., 2017).

For questions 1 and 2, where we explored the influence of temperature on overall aggression (overt and restrained aggression combined) and overt aggression and restrained aggression separately, we first scaled the counts of aggression to account for individual differences in aggression as expected from previous studies, where fish showed between-individual differences in metabolic response to temperature and aggressive behaviour (McKenzie et al., 2015; Metcalfe et al., 2016). Therefore, aggression counts per trial were divided by the observed maximum count of aggression for that fish in any trial. The final model for each response variable was selected using Akaike information criterion (AIC) comparison (see Table A1 for model comparison details). The scaled aggression response variable was fitted against trial temperature as a continuous nonlinear factor (from the temperature measures taken immediately after the trial). We included 'day' in the model as a nonlinear categorical factor as we expected aggression to vary with repeated exposure to the mirror test (Hotta et al., 2018). We also included 'Fish ID', which was a significant term in the models (see Results for more detail). We did not include trial as it did not account for significant variation in the model.

For question 3, we divided the data into distinct within-trial 5 min periods. We then fitted a similar GAM model as in question 1 to examine whether rates of overall aggression were sustained across trial lengths at different temperatures. Here we included the categorical factor 'Period', which had five levels, for each 5 min period of the trial.

Ethical Note

The experiments adhered to the ASAB/ABS Guidelines for the treatment of animals in behavioural research and teaching and procedures performed were in accordance with the ethical standards of, and approved by, the Manchester Metropolitan University Ethics Panel (FREGSE_UG/PGT_004_21.02.2022). One of the main sources of potential stress to the fish would have been transfer between tanks. Only experienced handlers caught the fish, and transfer time was kept to a minimum. Fish were monitored for at least 15 min immediately after any movement between tanks. We checked for colour changes (very pale or very dark) and behaviour changes that might indicate fish stress and a lack of wellbeing. Fish only showed minimal signs of stress and in all cases colour changes and sheltering ended within 15 min. All fish maintained a healthy appetite throughout the study. The other major welfare concern is aggression in the housing tanks. Here, careful monitoring was performed; if aggression was noticed between fish, the aggressive individual was separated and moved to a new tank. Clay pots were added to all tanks to provide shelter. Mirror tests and physical strikes with the mirror do not cause damage to this species, with no reports of damage from multiple previous studies. No damage was observed in the fish in our study either. All individuals were retained in the laboratory for use in future studies.

RESULTS

Effect of Temperature on Frequency and Type of Aggression

Aggression was overall significantly influenced by trial temperature (all GAM: $\text{edf} = 1.95$, $F_{1,9} = 9.821$, $P < 0.001$; Fig. 1). Similar significant relationships were observed in models that fitted overt (Overt GAM: $\text{edf} = 1.87$, $F_{1,9} = 3.346$, $P = 0.043$; Fig. 2) and restrained (Restrained GAM: $\text{edf} = 1.95$, $F_{1,9} = 10.543$, $P < 0.001$; Fig. 2) behaviours against temperature. The day of testing also had a strong influence on aggression: in the overall aggression model, trial nested within day as a factor had a nonlinear influence on temperature over consecutive days (all GAM: $\text{edf} = 8.01$, $F_9 = 7.900$, $P < 0.001$; Fig. A1). Individual differences in aggression were also important: across the 10 days of testing, individual repeatability 'R' in rates of overall aggression between fish was high ($R \pm \text{SE} = 0.748 \pm 0.073$, 95% confidence interval = 0.621–0.895, $P < 0.001$; Fig. A2).

Effect of Temperature on Aggression over Time

Rates of scaled overall aggression across time (periods of 5 min) within a trial were significantly influenced by temperature, with an interaction between temperature and period impacting levels of aggression across all periods (Fig. 3, Table 3). A similar influence of temperature was seen with predictions from the model with unscaled data (Fig. A3, Table A2).

DISCUSSION

By testing aggression over a comprehensive range of temperatures our study contributes to the understanding of fine-scaled effects of changing temperature on agonistic behaviour in a fish species. We demonstrated that aggression in *N. pulcher* varies with increasing temperature in a nonlinear manner with acute exposure and short acclimation to temperature. Building on previous work on aggression in other Tanganyika cichlids (Kua et al., 2020), we showed that the linear relationship with temperature only holds true for parts of the thermal range to which fish may be exposed, at least for

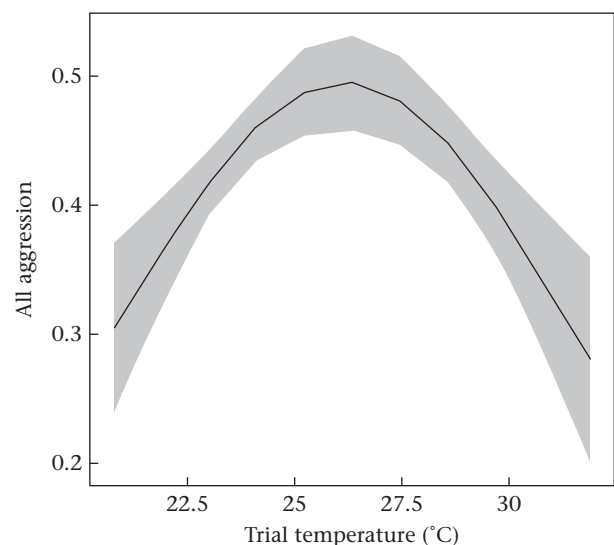


Figure 1. Frequency of aggression across a range of temperatures. The line depicts the predicted rate of aggression (scaled to the level of aggression per fish) across temperature from the fitted model. The band indicates the estimated 95% confidence interval smoother from the fitted model. The predictions were made with the terms Day and Fish ID excluded. $N = 158$ trials from eight fish.

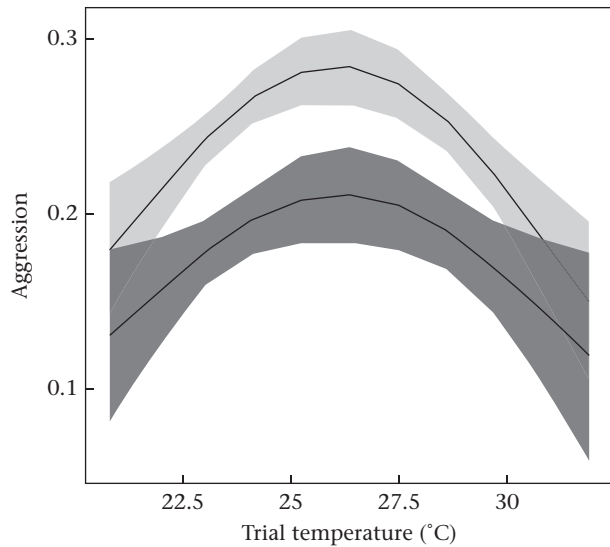


Figure 2. Frequency of both overt and restrained aggression across temperatures. The lines represent the predicted rates of aggression (scaled per level of aggression per fish) from the fitted model. The bands are smoothers showing the 95% confidence interval prediction from the fitted model for restrained (light grey) and overt (dark grey) aggression across temperatures. The predictions are made with the terms Day and Fish ID excluded. $N = 158$ trials from eight fish.

short term exposure and limited time for acclimation. This reflects the expectation given underlying physiological thermal performance curves. Our study also revealed that both overt and restrained displays of aggression are similarly affected by temperature. Exploring reactions towards the mirror within trials suggests that the drop in aggression at higher temperatures is driven by sharply decreasing rates of aggression during the course of a given trial. At high temperatures fish exhibited high levels of aggression initially, followed by rapid reductions over time: in the first 5 min of exposure to a mirror opponent aggression increased with increasing temperature, but at temperatures over 28 °C it decreased with increasing temperature after extended exposure to the mirror. This is mostly likely driven by more rapid physiological fatigue at these temperatures (Nilsson et al., 2009) where rate of aggression, or vigour, is affected by physiological performance. This is similar to, for example, hermit crabs where vigour is reduced in hypoxic conditions (Lane & Briffa, 2023). These nuances in the temperature and aggression relationship may have consequences in this, and other species, exposed to acute changes in thermal environment with more common extreme fluctuations expected from climate change (Phiri et al., 2023). However, on a longer timescale most fish species might be able to adapt to both increased temperatures and larger thermal variance (Crozier & Hutchings, 2014). Whether our study species specifically is able to adapt will depend on how close the temperatures they experience are to their thermal limits (Somero, 2010). In our study we also focused on fish from one population. It would be interesting to repeat the study with more individuals from different populations to test for the generalizability of our findings.

Tropical ectotherms are likely to be more sensitive to fluctuations in temperature and to more extreme temperatures due to their adaptation to more stable thermal ranges (Chevin et al., 2010; Huey et al., 2012; Morgan et al., 2022). This might be particularly important for species where aggression is critical for social organization and as an antipredator mechanism (Heg et al., 2004; Reddon et al., 2019). The direction of these changes is difficult to predict from our data and will depend on whether social ranking depends on relative or absolute levels of aggression and rates of attacks by predators, which are also affected by temperature. We might expect that increased aggressive intensity will lead to less stable social

groups, higher risk of injuries and reduced body condition due to higher energetic demands. On the other hand, the break-down of high levels of aggression in warmer water after a rather short period of time might indicate that at high temperatures fish will be less likely to engage in prolonged aggressive interactions, which might result in more stable groups, but also lowered levels of defence against predators. Predation risk is a key driver of sociality in several highly social fish species (Groenewoud et al., 2016; Josi et al., 2020; Tanaka et al., 2016), and predator behaviour is likely also to be impacted by temperature changes. Future studies may test the combined effects of temperature on social systems, predator attacks and defensive aggression in these systems. Potentially, short-term adjustment to high energetic costs of aggression in warmer conditions might cause fish to switch from overt aggressive behaviours, which are more physiologically costly (Neat et al., 1998), to extended phases of visual threat displays. However, in our data we did not find such an effect, with both overt and restrained aggression following similar patterns in response to temperature. An additional question worth considering is the influence of interindividual differences in aggression linked to interindividual physiological variation (Tanner & Dowd, 2019). For example, individual fish with higher stores of heat-shock proteins might be able to sustain levels of aggression at higher temperatures for longer bouts and might in turn be more likely to become dominant in future climate scenarios. For this, and other, follow-up research larger sample sizes of fish would be useful.

An important point to note in our study is that we used short-term exposure with relatively limited time for thermal acclimation (minimum of 16 h). Longer acclimation time for the fish prior to testing may influence the results. Indeed, the length of exposure time and acclimation periods are critical determinants of physiological responses to temperature (Schulte et al., 2011). The magnitudes of physiological, and consequently behavioural, responses to temperature may drop as populations adapt to increasing temperatures (Wootton et al., 2022). However, with increasing frequency and magnitude of thermal fluctuations due to climate change and associated environmental change (Paaijmans et al., 2013), the observed impact on aggression from short-term exposure to different temperatures is still important for understanding and predicting real world impacts of higher temperatures on aggression. It is also important to frame our results with respect to the critical thermal maximum (CT_{Max}) of our study species. The observed drop in aggression with increasing temperature may have been driven by approaching the CT_{Max} of *N. pulcher*. However, we did not observe any avoidance type behaviours that are typical of cichlids, and fish in general, that are exposed to temperatures close to their thermal maximum. Indication of thermal avoidance behaviour includes changes in swimming behaviour, such that fish start to swim around the edges of their aquaria in a quick burst of swimming. There were also no incidences of loss of equilibrium, one of the most common measures of thermal maximum in fish (Becker & Genoway, 1979; Brandão et al., 2018; Christensen et al., 2021). Other tropical cichlids that have been tested (specifically *Pseudocrenilabrus multicolor*) reached CT_{Max} at above 38 °C (McDonnell & Chapman, 2015).

From a methodological perspective, these results provide several points to consider. First, they reinforce previous calls for careful temperature control and selection in measuring behavioural responses to temperature (Biro et al., 2010; Forsatkar et al., 2016; Killen et al., 2013). Previous work has shown that measures of boldness and activity can depend on acute exposure to fine-scale differences in temperature in fish (Biro et al., 2010). Similarly, levels of aggression in other ectotherms such as ants can depend on small temperature differences (Krapf et al., 2022). Second, our results highlight the importance of the duration of trials of aggression. In short trials the levels of aggression may appear to increase with temperature; however, over longer trials a drop in aggression

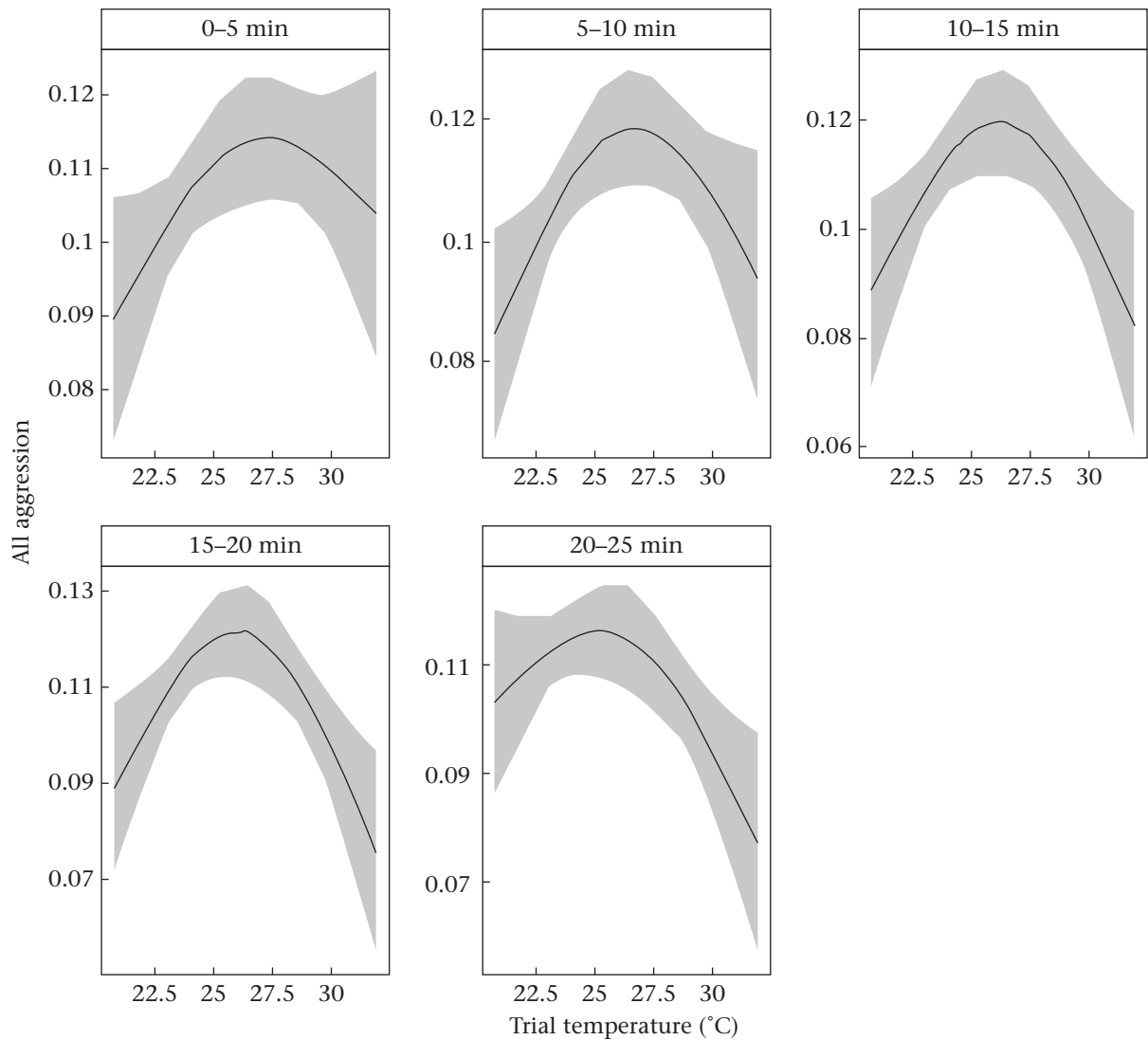


Figure 3. Changes in frequency of scaled aggression over time within a trial across temperatures. The black line denotes the estimate of aggressive behaviour plotted against the trial temperature in each panel. Each panel is the estimated level of aggression per 5 min period of the 25 min trials. The grey band is the estimated 95% prediction smoother given the fitted model. The predictions are made with Day excluded. $N = 158$ trials (two trials per day) for 10 days for each of eight fish.

becomes apparent. Both these points are important for individual studies, but also for general comparison between studies. Our results may provide an explanation for unexpected findings in past work. For example, in eastern mosquitofish, *Gambusia holbrooki*, there was no difference in rates of aggression between individuals exposed to high and low temperatures (Matthews & Wong, 2015). This somewhat surprising finding may have been driven by the two

Table 3
Results of the generalized additive model showing the effects of temperature on aggressive behaviours scaled to level of aggression per fish across the duration of a trial, broken into 5 min periods of time

Approximate significance of smooth terms				
	edf	df	F	P
s(Temp):Period 0–5 min	1.717	1.92	2.291	0.157
s(Temp):Period 5–10 min	1.861	1.981	3.712	<0.032
s(Temp):Period 10–15 min	1.891	1.988	4.049	0.017
s(Temp):Period 15–20 min	1.912	1.992	5.310	0.005
s(Temp):Period 20–25 min	1.822	1.968	3.380	0.025
s(Day)	8.985	9	27.178	<0.001

Intercept: estimate \pm SE = 0.106 \pm 0.009, $t = 10.750$, $P < 0.001$.

temperatures used; the high and low temperatures of that study were 22 and 32 °C, respectively (matching the temperatures associated with lower aggression in our study). If *Gambusia* have similar thermal ranges and underlying thermal performance curves to *N. pulcher*, then their measures of aggression would have been sampled at two extreme points of the performance curve. However, unlike our study species *Gambusia* have a wide distribution and high thermal tolerance, so their thermal performance may not be comparable to that of our fish. Relatively lower rates of aggression at very high temperatures have been found in other tropical fish as well, such as coral reef species where aggression rates at 34 °C were lower than those at 28 °C (da Silva-Pinto et al., 2020).

While not the main focus of our study, we found that increasing numbers of trials across consecutive days resulted in significant changes in aggression. Rates initially increased in the first few days and then progressively decreased during the latter part of the experiment. This reduction might indicate that the fish learned to recognize themselves in the mirror, as has been shown for cleaner wrasses (Kohda et al., 2019, 2022). However, a study on *N. pulcher* revealed no evidence for mirror self-recognition (Hotta et al., 2018). More likely, the reduced amount of aggression might be explained

by dear enemy effects (Frostman & Sherman, 2004), or by a general habituation to the 'odd' behaviour of a mirror opponent (Brown & Noakes, 1974; Desjardins & Fernald, 2010; Meliska & Meliska, 1976). These results hence call for caution and careful validation of repeat testing of fishes in mirror test studies of aggression.

In summary, this study shows that aggression, both the overall frequency and the intensity over time, is influenced by temperature, with potential implications for interactions that are mediated by aggression in this and other ectotherm species. It also highlights methodological implications for future work in this area, where temperature and assay duration can influence measures of aggression. Our results will hopefully aid future research by highlighting the importance of testing aggression across a full range of temperatures, and considering the potential pitfalls inherent in comparing aggressive behaviours across limited numbers of specific temperatures.

Author Contributions

N.A.R.J. and J.G.F. conceived of the study; N.A.R.J., J.N.-Y. and J.G.F. planned the experiment; J.N.-Y. and J.G.F. cared for the animals; N.A.R.J. and J.N.-Y. established the set-up; N.A.R.J. ran the experiments, analysed the videos, conducted the statistical analyses and drafted the manuscript; J.N.-Y. and J.G.F. worked on the drafts.

Data Availability

A full data set and the code can be found at the Open Science Framework at https://osf.io/dgkyz/?view_only=677654a321cc4cb4aea7634d655d3c0d.

Declaration of Interest

The authors declare no conflict of interest.

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Supplementary Material

Supplementary material associated with this article is available, in the online version, at <https://doi.org/10.1016/j.anbehav.2023.10.008>.

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Appendix

Table A1

Model details and Akaike information criterion (AIC) scores for comparing model fits

Model name	Model	df	AIC	Deviance	
Aggression (Scaled by Fish) across temperature	gam(ScaledAggression ~ s(Temp, k = 3, bs = 'tp') + FishID + s(Day, bs='re'))	19.32517	-61.8891	51.5	Selected
	gam(ScaledAggression ~ s(Temp, k = 4, bs = 'tp') + FishID + s(Day, bs='re'))	19.97559	-60.7772	51.6	
	gam(ScaledAggression ~ s(Temp, k = 3, bs = 'tp') + FishID + Trial + s(Day, bs='re'))	20.32042	-59.8863	51.5	
	gam(ScaledAggression ~ s(Temp, k = 5, bs = 'tp') + FishID + s(Day, Trial, bs='re'))	26.39946	-51.6052	52.7	
	gam(ScaledAggression ~ s(Temp, k = 5, bs = 'tp') + s(Day, Trial, bs='re'))	12.13741	-31.901	35.7	
Aggression (Scaled by Fish) across time 'Period' (within trial) and temperature	gam(ReScaledAll ~ s(Temp, bs = 'tp', by = Period, k = 3) + s(Day, bs='re'))	23.89907	-2402.58	29.4	Selected
	gam(ReScaledAll ~ s(Temp, bs = 'tp', by = Period, k = 4) + FishTag + s(Day, Trial, bs='re'))	28.84318	-2471.55	36.4	
	gam(ReScaledAll ~ s(Temp, bs = 'tp', by = Period, k = 3) + FishTag + Trial + s(Day, bs='re'))	28.92506	-2509.87	39.4	
Aggression across time 'Period' (within trial) and temperature	gam(All ~ s(Temp, bs = 'tp', by = Period, k = 4, m = 1) + FishTag + s(Day, bs='re'))	31.8766	17661.42	47.3	Selected
	gam(All ~ s(Temp, bs = 'tp', by = Period, k = 3, m = 1) + FishTag + s(Day, bs='re'))	26.99778	17706.28	47	
	gam(All ~ s(Temp, bs = 'tp', by = Period, k = 4, m = 1) + FishTag + s(Day, Trial, bs='re'))	32.85488	18341.5	44.5	

Table A2

Results of the generalized additive model on levels of aggression, unscaled, showing the effects of temperature on aggressive behaviours across the duration of a trial, divided into 5 min periods of time

Approximate significance of smooth terms				
	edf	df	χ^2	P
s(Temp):Period 0–5 min	2.912	3	174.8	<0.001
s(Temp):Period 5–10 min	2.94	3	211.2	<0.001
s(Temp):Period 10–15 min	2.92	3	158.9	<0.001
s(Temp):Period 15–20 min	2.932	3	183.8	<0.001
s(Temp):Period 20–25 min	2.915	3	187.7	<0.001
s(Day)	8.985	9	5092.0	<0.001

Intercept: estimate \pm SE = 4.445 \pm 0.104, $z = 42.650$, $P < 0.001$.

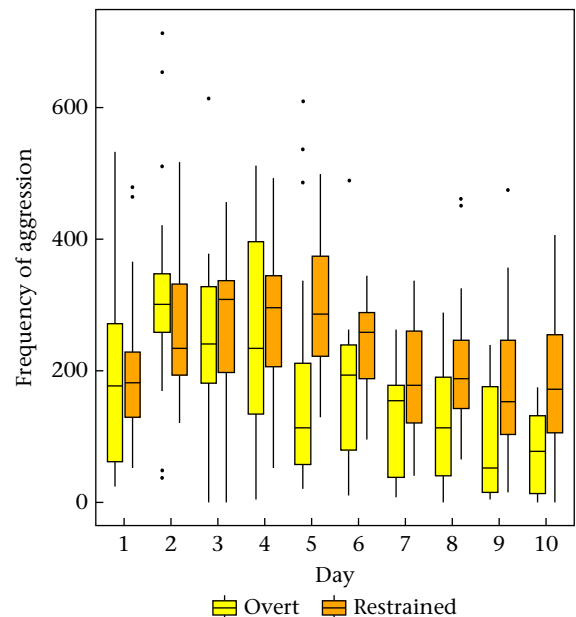


Figure A1. Counts of both overt and restrained forms of aggression across days of testing. Box plots show the median, 25th and 75th percentiles; the whiskers extend to the data point that is no more than 1.5 times the length of the box as per the Tukey method and black circles are outliers.

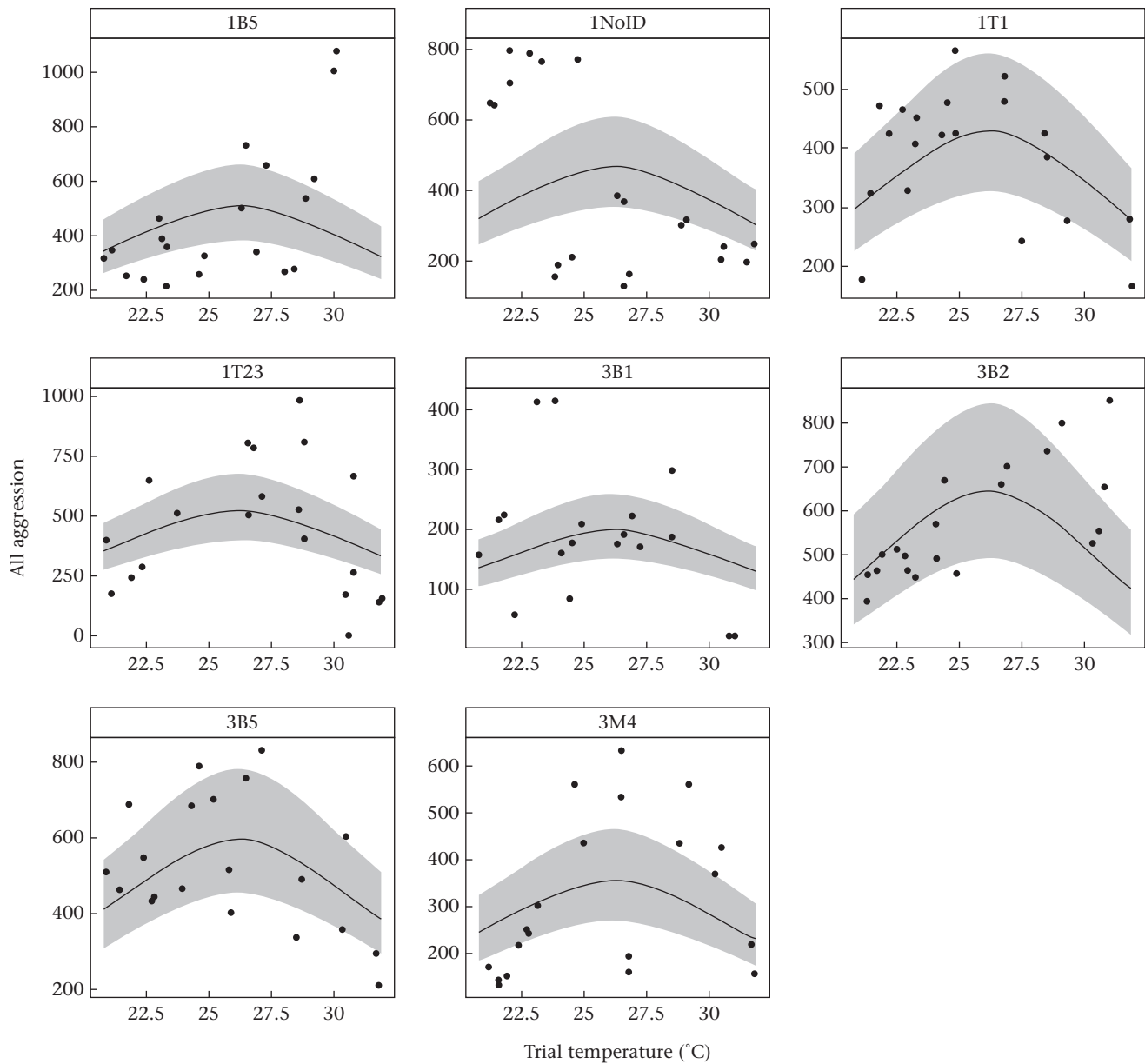


Figure A2. Model predictions of total aggression across temperatures for each fish tested (the fish ID is given above each panel). The black line denotes the estimate of aggressive behaviour plotted against the trial temperature in each panel. Predictions are from a model fitted with total aggression without scaling. The grey band is the estimated 95% prediction smoother given the fitted model. The predictions are made with Day excluded. $N = 158$ trials (two trials per day) for 10 days for each of eight fish.

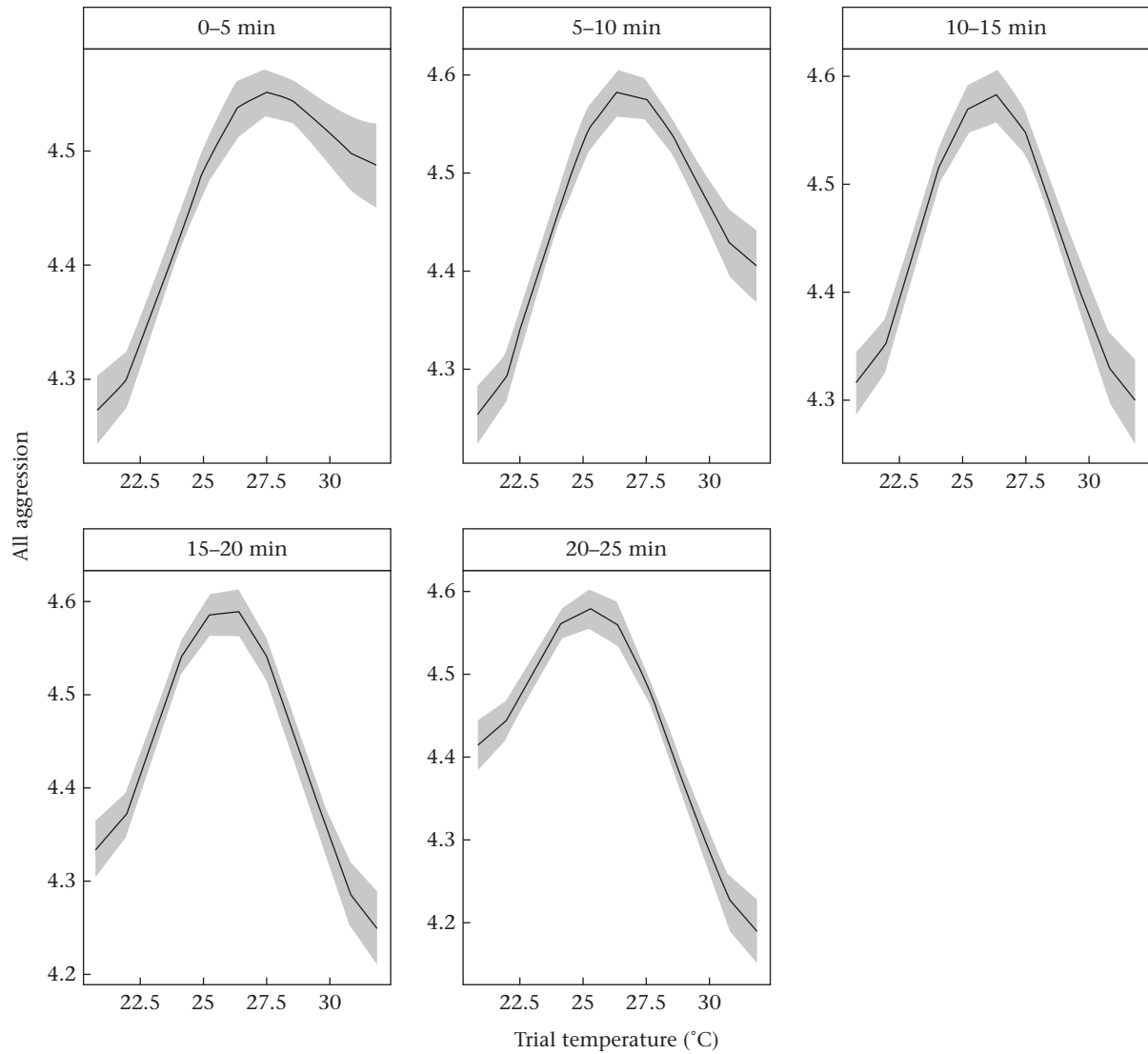


Figure A3. Frequency of unscaled aggression over time within a trial across temperatures. The black line denotes the estimate of aggressive behaviour plotted against the trial temperature in each panel. Each panel is the estimated level of aggression per 5 min period of the 25 min trials. The grey band is the estimated 95% prediction smoother given the fitted model. The predictions are made with Day excluded. $N = 158$ trials (two trials per day) for 10 days for each of eight fish.