Title: The reproductive seasonality and fecundity of yellowtail clownfish (*Amphiprion clarkii*) in the Philippines

Running title: Reproduction in yellowtail clownfish

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ABSTRACT

Understanding the spawning patterns and egg production of clownfish is important for understanding their life history and the factors contributing to population persistence. The egg production and temporal spawning patterns of eight breeding pairs of yellowtail clownfish, *Amphiprion clarkii* (Bennett 1830), were observed for a 14-month period on a coral reef in the Central Visayas, Philippines. Spawning events and egg production revealed a peak breeding season from November through May, which coincides with temperatures below 30 °C. Noticeably fewer spawning events and smaller clutch sizes occurred during the warmer months (30 to 31.5 °C) of June through October. Within the spawning season, egg production increased weakly leading up to the new moon and decreased after the full moon. The seasonality of spawning events found in this study were comparable to those of clownfish in temperate regions, and surprisingly unlike findings from other tropical latitudes and climates. These findings suggest that recruitment and larval dispersal in this population will be most sensitive to oceanographic conditions during relatively narrow periods of time each year.

INTRODUCTION

Population stability depends on the net replacement of individuals across generations (Hastings & Botsford 2006). Net replacement, however, can be accomplished with a wide range of reproductive strategies, including from rare production of many offspring to frequent production of few offspring (Roff 1984; Winemiller & Rose 1992). In the ocean, many species produce large numbers of offspring, often with a distinct seasonal pattern to reproduction (Johannes 1978; Winemiller & Rose 1992). Water temperature is commonly identified as a major factor that determines a species' spawning season (Righton et al. 2010), possibly because developing eggs and larvae often have narrow temperature requirements for normal development (Rijnsdorp et al. 2009). Alternatively, spawning cycles may be timed to take advantage of specific current and wind patterns that disperse eggs or larvae with minimal loss of offspring (Lobel 1989). However, fishes are often assumed to lack seasonal spawning patterns in the tropics, possibly due to the narrow seasonal range in water temperatures (Allen 1972; Ross 1978).

Within spawning seasons, reproduction often displays a lunar pattern, and spawning activity and egg production may be greater during specific phases of the moon (Johannes 1978; Pressley 1980; Samoilys 1997; Madhu & Madhu 2007; Helfman et al. 2009; Madhu & Madhu 2012). A lunar spawning cycle can benefit both offspring and adults, though benefits vary among species (Johannes 1978). For example, offspring survival may be higher during large tidal magnitudes associated with the moon, which can increase the dispersal of pelagic larvae away from spawning areas where predators may be abundant (Robertson et al. 1990). Adults may also have adopted a lunar spawning cycle in order to synchronize breeding among individuals while allowing for sufficient recuperation between spawning events (Robertson et al. 1990). This recuperation period may be especially important for benthic spawning fishes because they typically incur the energetic cost of prolonged parental care (Helfman et al. 2009). Alternatively, ease of nest care or food availability can vary on a lunar cycle (Johannes 1978; Pressley 1980).

The yellowtail clownfish (*Amphiprion clarkii*) is a benthic spawning reef fish that is common to the tropical and subtropical Pacific Ocean (Fautin & Allen 1992). *Amphiprion clarkii* possess the two-stage life cycle common to many reef fishes, consisting of a relatively sedentary juvenile and adult stage, and a pelagic larval stage that allows for wide-scale dispersal (Burgess et al. 2014; Pinsky et al. 2010). Clownfish live in close association with anemones, which they can use as protection from predators because clownfish are not affected by the stinging nematocysts (Fautin & Allen 1992). Further, clownfish exhibit a high level of site fidelity with specific anemones (Fautin & Allen 1992). They typically do not move more than a few meters from anemones, making clownfish ideal model species for individual-scale studies on reproduction (Fautin & Allen 1992). Occupied anemones are typically composed of one dominant breeding pair of clownfish and up to four nonbreeding males (Fautin & Allen 1992). *Amphiprion clarkii* are protandrous sequential hermaphrodites, meaning that they first develop as males (Ochi 1989). When the female is lost from an anemone, it is replaced by a sex-changing male (Fautin & Allen 1992). Breeding adult clownfish are easily visible and distinguishable from non-breeders because caudal fin coloration depends on sex and stage of maturity (Hattori & Yanigasawa 1991). During spawning, females lay a single layer of eggs on a hard substrate near the host anemone (Fautin & Allen 1992).

The purpose of this study was to investigate the reproduction and fecundity of *A. clarkii* in the tropical waters surrounding Leyte, Philippines, in order to determine whether there were seasonal and lunar breeding patterns and to estimate the total number of eggs produced seasonally and annually. Determining when clownfish produce eggs and how many they produce will be helpful for understanding how these populations replenish themselves, including when the dispersal and survival of their larval offspring will be most sensitive to oceanographic conditions.

MATERIALS AND METHODS

From February 2015 to March 2016, all anemones that hosted clownfish were visited on the fringing coral reef adjacent to Visayas State University in Baybay City, Leyte, Philippines (10.743° N, 124.786 °E). The site was chosen for ease of access from the University, since the project involved daily surveys. The reef had low relief (< 1 m) without a distinct reef flat or slope, extended 290 m from north-northeast to south-southwest, and was 70 m wide. The depth on the reef was 2 to 5 m. Sand or muddy bottom were found beyond the offshore, northern, and southern reef edges. The clownfish were found throughout the reef and primarily inhabited anemone species *Heteractis crispa* (Hemprich & Ehrenberg in Ehrenberg 1834), but one pair was found on each of *Stichodactyla mertensii* (Brandt 1835) and *Macrodactyla doreensis* (Quoy & Gaimard, 1833). Distances to each pair's nearest neighbor ranged from 25 to 55 m.

From February 2015 through July 2015, five anemones with breeding pairs were present in the sampling area (Table 1). Two more breeding pairs were discovered on other anemones on July 15. On August 25, one of the original anemones disappeared and a new anemone was discovered (Table 1). While this may indicate anemone movement, the breeding pair present on the new anemone was visibly smaller than the previous pair on the now-disappeared anemone (7 and 6 cm instead of 11 and 10 cm) and was therefore treated separately.

Six days a week, two divers visited each of the anemones to determine the presence or absence of *A. clarkii* breeding pairs and their eggs on each anemone (except for 69 days missed for storms or other events). In this population, non-breeders typically have transparent or rounded yellow fins, adult males typically have caudal fins with orange borders, and females typically have pointed yellow caudal fins. If eggs were present around the anemone, a video and photos of the eggs were taken with a set of calipers held by the clutch for sizing. The length and width of each clutch was measured and the egg color and other features were recorded.

Two HOBO U22 Water Temp Pro v2 water temperature loggers (Onset Computer Corporation, Bourne, MA) were also deployed 80 m offshore at a depth of 4 m to take temperature readings at 30-minute intervals, offset from each other by 15 minutes. The temperature readings were averaged within each day to calculate a mean daily temperature. In May 2015, the females were caught and measured with calipers to the nearest mm.

The best photo on the first day of each clutch was analyzed using ImageJ software to determine the number of eggs (Schneider 2012). The calipers in the image were used to set the scale, and eggs were counted within a 1cm x 1cm square sample within each clutch. The major (A) and minor (B) axes of the clutch were measured, and the area of each clutch was then calculated as an ellipse: $\pi \times A \times B$. The total number of eggs per clutch was calculated as the product of density (eggs/cm^2) and area (cm^2).

To test hypotheses about the temporal patterns of *A. clarkii* spawning, we fit two generalized additive mixed effects models (GAMMs) using the gamm4 package in R software version 3.2.4 (R Core Team 2016; Wood & Scheipl 2016). We used GAMMs because, like generalized additive models (GAMs), they allow the response variable to be a non-linear smooth function of the predictor variables, and so could capture seasonal and lunar cycles or non-linear responses to temperature (Wood & Scheipl 2016). Beyond GAMs, however, GAMMs allow the inclusion of random effects in the model (Wood & Scheipl 2016). Random effects were helpful for accounting for the correlated error structure in our dataset, which was produced by repeated (daily) observations from each clownfish pair.

The first GAMM was for the occurrence of spawning. In this model, each observation was for a clownfish pair observed on a particular day, and the response variable was whether that day was (1) or was not (0) the first day we observed a new clutch for that clownfish pair. We specified a binomial error structure since the response variable was binary. The second GAMM we fit was for the number of eggs in a clutch, conditional on a clutch having been laid. In this model, the response variable was log(number of eggs) and each observation was a unique clutch from a clownfish pair. This model had Gaussian errors.

Predictor variables for both models included the day of the year, day of the lunar month, water temperature (°C), days since the last clutch was laid, and female size (cm). We fit smooth terms for all variables except for female size. The relatively low numbers of observed females forced us to use a linear term for female size instead of a smooth term. To account for the fact that we had repeated observations from each pair of fish, we included breeding pair ID as a random effect in both models. Including random effects helps correct for the correlated error structure in hierarchical datasets like ours (Zuur 2009).

The eggs produced per pair of fish per day depends both on the probability of spawning and on the size (in eggs) of the clutch produced if they do spawn. For visualizing temporal patterns in eggs per pair per day, we therefore combined the two models' predictions as $\hat{\square} = \hat{\square} \times \square^{\hat{\square}} \times \square$, where $\hat{\square}$ was our estimate of eggs produced per pair per day, $\hat{\square}$ was the first model's estimate of the probability of spawning, $\hat{\square}$ was the second model's estimate of log(clutch size), and □ was Duan's smearing estimator to help correct for retransformation bias in the clutch size model (Duan 1983).

Because we only observed one annual cycle, we cannot be sure whether other years follow similar cycles. However, our supplementary observations of nest presence and absence in June 2014 (absence), January 2015 (presence), and May and June 2016 (absence) suggest broadly similar patterns across multiple years.

RESULTS

Temporal spawning patterns

The time between spawning events was highly variable (7 to 195 days), in part because most breeding pairs did not spawn during the warm season from June through October (pairs 776 and 1284 laid one and two clutches, respectively, during these months). Median time between spawning events was 16 days. The maximum number of clutches over the course of 14 months was 14 (Table 1). Eggs typically hatched after 6 days (range 5 to 8 days) after transitioning through bright red, orange, purple, and then silver colorations.

The average number of spawning events was the highest in the month of March, and no spawning occurred in July and September (Fig. 1A). Spawning generally occurred when water temperatures were below 29.5 °C, and few spawnings occurred at temperatures greater than 29.5 °C (Fig. 1A).

Spawning also varied across the lunar month. Spawning frequency was highest on day 29 (the day before the new moon) and somewhat elevated on days 7-13 leading up to the full moon (Fig. 2A). There was also a small decrease in spawning events directly after the new moon (days 3-6) and after the full moon (days 15-23), with relatively few spawning events until the next new or full moon approached (Fig. 2A).

When analyzed in a statistical framework that considered season, lunar cycles, temperature, and previous spawning, the model suggested that temperature and days since last spawn were the two statistically significant factors determining spawning patterns (Table 2). Spawning probability in the statistical model peaked around 29 °C and was lowest immediately after a previous spawning event (Fig. 3C and D).

Fecundity

The average clutch size was 1,763 ± 96 eggs (expressed as ±1 standard error) (Table 1). Among the breeding pairs that were observed for a full year (or for the full study), the average number of eggs per clownfish pair per year was 14,433 ± 3,431 (or 16,546 ± 3,067 over the 14-month study) (Table 1). Breeding pair #1338 produced the most eggs for the year (28,798 eggs), had the highest average clutch size (2400 ± 199 eggs), and contained the largest female (11.9 cm) (Table 1). Egg production across all eight breeding pairs was the highest during the months of November through May (Fig. 1B), highest just around the new moon (days 0 and 29), and somewhat higher in the period before the full moon (days 7-13) as compared to the period after the full moon (Fig. 2B).

The statistical analysis suggested that clutch size, conditional on a clutch being laid, was correlated to lunar day, temperature, day of the year, and days since the previous spawning event (Table 2). Clutch size increased but not significantly with female size, possibly because our dataset only had six measured females across a narrow range of sizes. The fitted smooth terms in the model suggested that clutch sizes were the largest in the months of December through April, largest around the new moon, and largest at temperature of 29 to 30 °C (Fig. 3).

When we combined predictions for the probability of eggs being produced and for clutch size, total numbers of eggs produced appeared highest in January through May, at temperatures below 29.5 °C, during lunar days above 25 and below 5 (around the new moon), and at least 20 days after the previous spawning event (Fig. 3).

DISCUSSION

Reproductive output is an important factor for understanding population persistence and coral reef fish life history strategies, both of which can be used to determine the effectiveness of marine protected areas and resource management plans (Burgess et al. 2014). This study aimed to define the temporal scale and fecundity of an *A. clarkii* population in the central Philippines by examining seasonal and lunar breeding patterns and egg production. Over the 14 month observational study, reproduction followed a clear seasonal pattern, with a peak during November through May when temperatures were below 29.5 °C. Less distinct peaks in spawning and egg production appeared to occur around the new moon and around the full moon.

While the Philippines has a tropical climate, these reproductive patterns are more similar to temperate populations with a specific breeding season, which is quite different from what has previously been seen in tropical anemonefish populations (Moyer 1980). For example, in the temperate waters surrounding Miyake-Jima, Japan, *A. clarkii* also have a distinct breeding season, but it has nearly the opposite timing and occurs from May through October when water temperatures are relatively high (Moyer 1980). However, the temperature at which spawning occurs in both these populations is similar, ranging from 22 to 29.5 °C in Japan and from 27 to

29.5 °C in our study from the Philippines (Moyer 1980; Bell 1976). Also in a temperate climate, *A. clarkii* in Murote Beach, Japan, began spawning in June as temperatures rose above ~20.5°C and ended at the beginning of October when temperature declined below ~22.5°C, which is similar seasonality to the population at Miyake- Jima, but at somewhat colder water temperatures (Ochi 1985). In Hong Kong, an area situated at an intermediate latitude between Japan and the Philippines, the first batch of eggs was found in April and reproductive activities continued through late September when the temperatures ranged from 22 to 28.5 °C (Leung 2000). Similar plasticity in reproductive seasonality has also been observed in other species (Righton et al. 2010), and it suggests that temperature is a widespread factor that influences reproduction in fishes (Rijnsdorp et al. 2009). In contrast, however, other tropical clownfish have been recorded to spawn all year long, including *A. melanopus* (Bleeker 1852) in Guam (13.4 °N), and *A. chrysopterus* (Cuvier 1830), *A. melanopus*, and *A. perideraion* (Bleeker 1855) in Eniwetok (11 °N) (Allen 1972; Ross 1978). Compared to other clownfish, our Philippines study population appears unique in having a seasonal spawning pattern with highest activity during the colder months.

Beyond seasonal patterns, the lunar cycle also appeared to weakly affect when spawning occurred within the reproductive season in the Philippines. A peak in spawning activity occurred the day before the new moon and there was little spawning activity during the 9 days following the full moon. Somewhat increased spawning frequency was also apparent during the week before the full moon. *Amphiprion clarkii* have an incubation period of around 6 days in the central Philippines, and spawning before the full moon may represent an occasional second clutch following a short recuperation period from the peak in reproduction around the new moon. This pattern is similar to *A. melanopus* in Guam (13.4 °N), which had spawning activity peaks near the first and third quarters of the lunar cycle and consequently, hatching near the full and new moons (Ross 1978). The lunar spawning patterns of *A. akindynos* (Allen 1972) in southern Australia (28.6 °S) are also similar, with increased spawning around the full moon (Richardson et al. 1997). However, while many anemonefishes display lunar patterns, these patterns can also be quite different from *A. clarkii* in the Philippines. For example, *A. latezonatus* (Waite 1900) in southern Australia (28.6 °S), as well as *A. percula* (Lacepède 1802) and *A. sandaracinos* in the Andaman Islands (10.8 °N), all have less frequent spawning in the days before the full moon (Richardson et. al 1997; Madhu & Madhu 2007; Madhu & Madhu 2012). Higher spawning for these populations occurs in the days just after the new or full moon (Richardson et. al 1997; Madhu & Madhu 2007; Madhu & Madhu 2012).

Potential explanations for lunar spawning periodicity include lower predation risk for larvae during stronger and higher spring tides, greater ease of nest care and predator avoidance during the full moon, higher levels of planktonic food sources for larvae during the full moon, or as a signal to entrain spawning synchrony to swamp predator feeding (Korringa 1947; Johannes 1978; Ross 1978; Pressley 1980). While our research was not designed to test among these explanations, the fact that *A. clarkii* in the Philippines have higher spawning activity around both the full and the new moon suggests that light or food availability are not primary factors. The weak increase in spawning before the full moon could be timed so that hatching six days later occurs during the larger tidal currents and deeper tidal depths of the spring tides, providing more rapid escape for larval fish from benthic and reef-associated predators. Spawning the day before the new moon, however, would not capture a spring tide effect, and may instead relate to benefits for the adult, possibly around nest care or predator avoidance. Alternatively, the lunar cycle could simply be a convenient cue for inducing spawning synchrony to reduce predation.

Both the average number of eggs produced in each clutch and the annual fecundity (Table 1) were similar to temperate populations of A. *latezonatus* in southern Australia (28.6 °S) and *A. clarkii* in Japan (34.1 °N) (Richardson et. al 1997; Bell 1976). In Japan, 8,000 to 17,500 eggs were produced annually, which is comparable to the average 21,000 eggs/pair/year in the Philippines. However, this is higher than many annual fecundity estimates in tropical waters, including 7,200 eggs for *A. melanopus* in Guam (13.4 °N), 6,000-9,600 eggs for *A. sandaracinos* in the Andaman Islands (10.6 °N), and 2,500-13,500 eggs for *A. percula* in the Andaman Islands (Ross 1978; Madhu & Madhu 2007; Madhu & Madhu 2012). In Hong Kong (22.2° N), the clutch size of *A. clarkii* ranged from 462 to 2,612 eggs (similar to the average 1,763 eggs in the Philippines), but annual fecundity was 3,321 to 12,373, which is somewhat lower than what was observed in Japan and the Philippines (Leung 2000; Richardson et. al 1997).

While living at a tropical latitude with water temperatures similar to that of other tropical areas where *Amphiprion* have been studied, reproduction in *A. clarkii* on Leyte, Philippines, also showed similarities to temperate anemonefish populations. The Leyte population had a lunar spawning pattern similar to that of tropical anemonefish and a distinct breeding season similar to temperate populations. Further, fecundity and clutch sizes were more similar to that of temperate anemonefish.

To understand whether reduced spawning during the warmest months is caused by temperature or by another correlated factor, additional years of observations in Leyte, Philippines or an experiment in an aquarium would be useful. In addition, studies of reproduction in other tropical anemonefish populations are needed to understand whether seasonal patterns are common throughout the tropics and whether reproduction is consistently rare during the warmest ocean temperatures. At a fundamental level, persistence depends on populations replacing themselves through sufficient reproduction. By understanding patterns of reproduction and their variation across space and time, we gain insight into the variety of strategies that help organisms cope with and persist through the variability in their environment. Understanding peak spawning times is also useful for designing future studies of development or larval recruitment, and for understanding when oceanographic conditions will most directly influence larval dispersal and survival.

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TABLES

Table 1. Data on each *Amphiprion clarkii* pair, including female fish length, number of days observed, number of clutches, average number of eggs produced per clutch (± 1 standard error), and the total number of eggs produced.

Pair ID	Female length (cm)	Duration	Begin	End	Clutches	Eggs per clutch	Eggs produced
776	9.8	392	2/5/15	3/3/16	14	1,485 ± 139	20,793
801	11	201	2/5/15	8/25/15	4	1,197 ± 215	4,788
1284	9.1	392	2/5/15	3/3/16	7	1,686 ± 182	11,805
1338	11.9	392	2/5/15	3/3/16	12	2,400 ± 173	28,798
1285	9.9	392	2/5/15	3/3/16	13	1,736 ± 199	22,573
n1	9.7	232	7/15/15	3/3/16	1	1,156	1,156
n2	—[†]	232	7/15/15	3/3/16	0	—	—
n3	—[†]	191	8/25/15	3/3/16	1	—[*]	—
Average ± 1 SE	10.2 ± 0.4	303 ± 11			12 ± 1.6[‡]	1,763 ± 95	20,993 ± 3,510[‡]

[†] For breeding pairs n2 and n3, the female was not caught and measured.

[*] For n3, the picture of the single clutch spawned was of low quality and the eggs could not be counted.

[‡] Calculated for pairs observed for the full study duration.

Table 2. Results from statistical analysis of spawning occurrence and clutch size with generalized additive mixed effects models (GAMMs). Coefficient is shown for the linear term, estimated degrees of freedom (DF) are shown for smooth terms, and estimated p-values are shown for all terms. Smooth terms are graphed in Fig. 3.

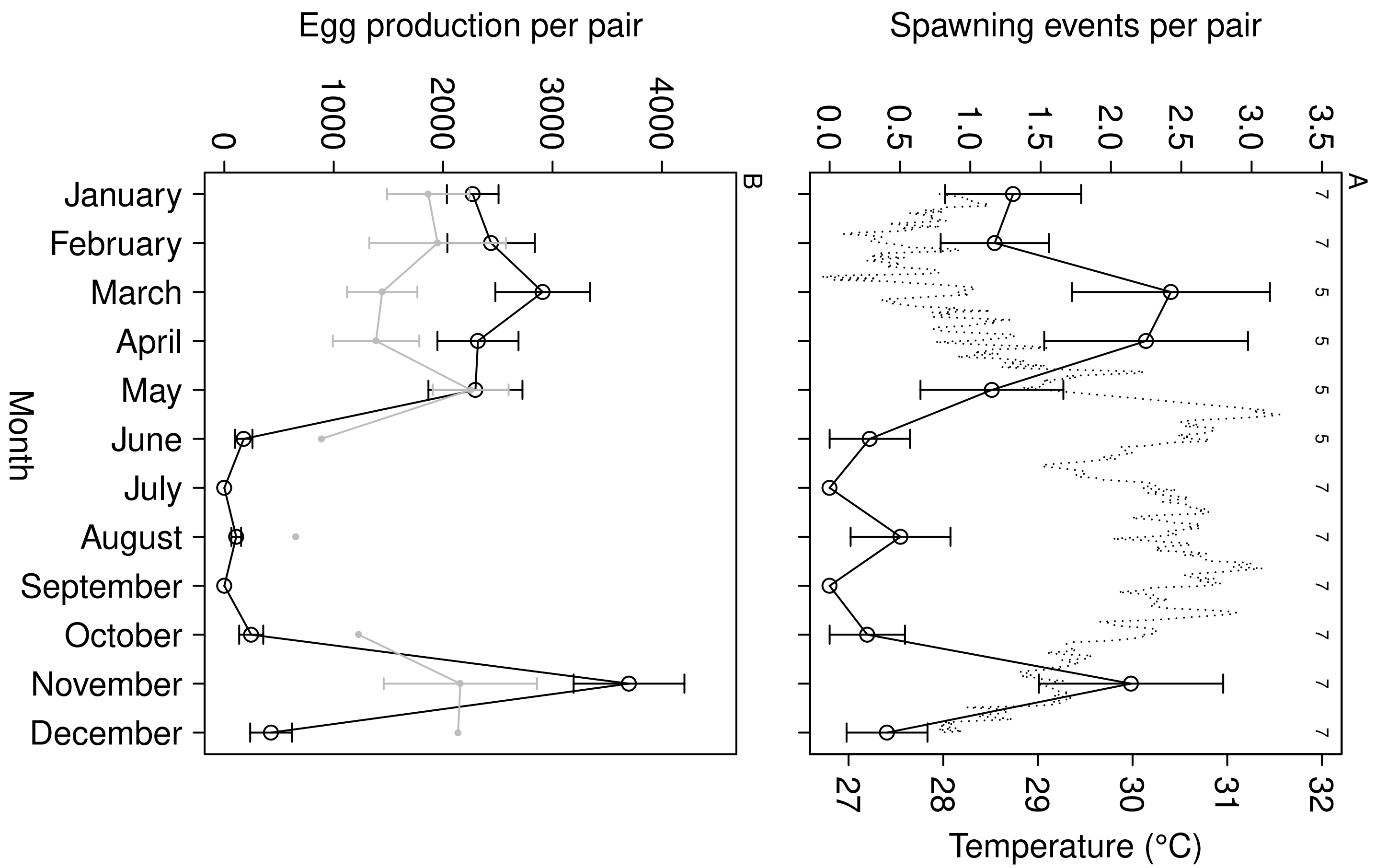
	Spawning model			Clutch size model		
Term	Coef.	DF	p-value	Coef.	DF	p-value
Lunar day		2.00	0.058		3.14	0.0022
Day of year		1.00	0.068		3.01	0.0046
Days since last spawn		2.22	0.011		1.00	0.017
Temperature		3.69	0.00096		3.58	0.011
Female size	-0.06		0.70	0.15		0.11

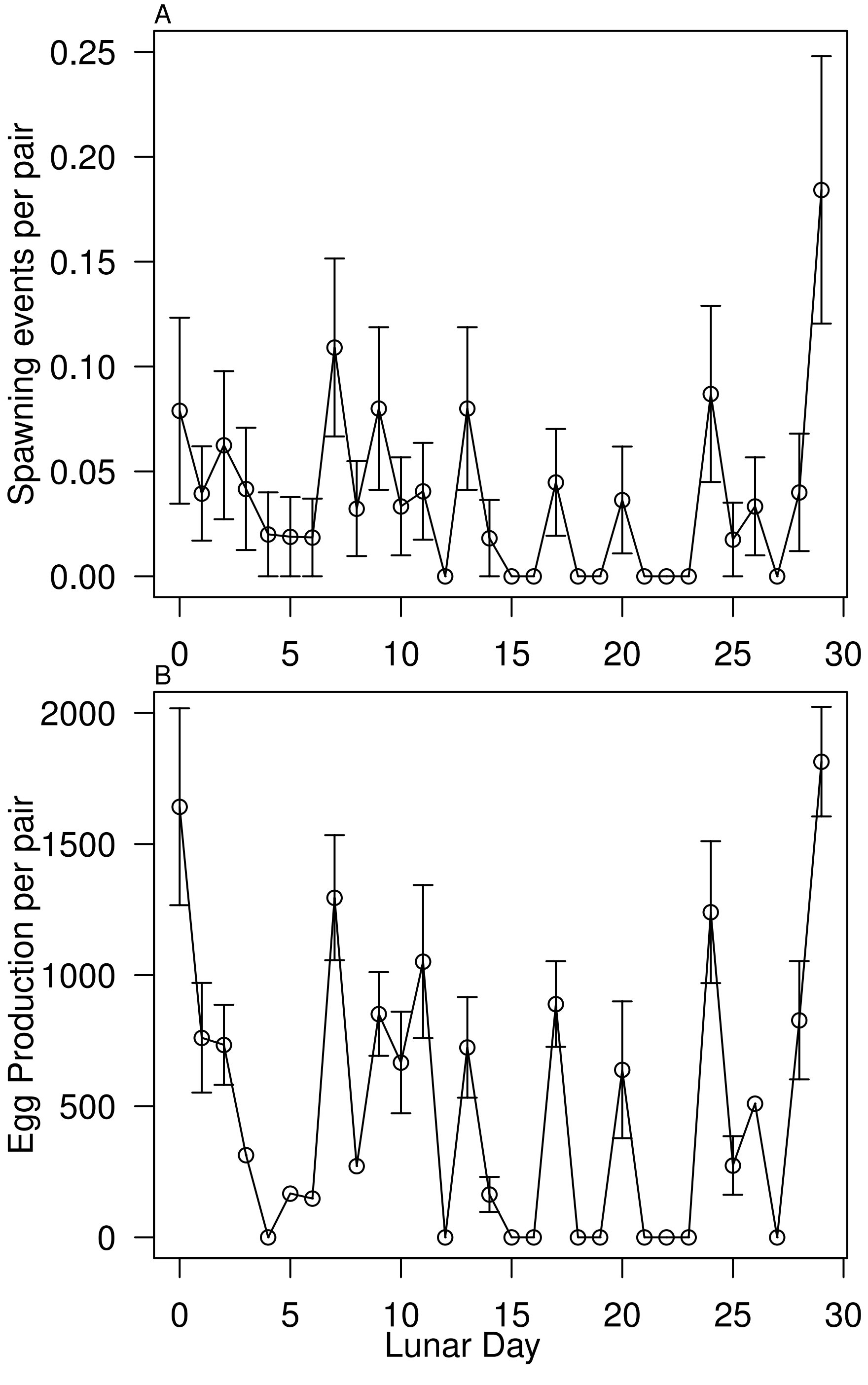
Figure Legends

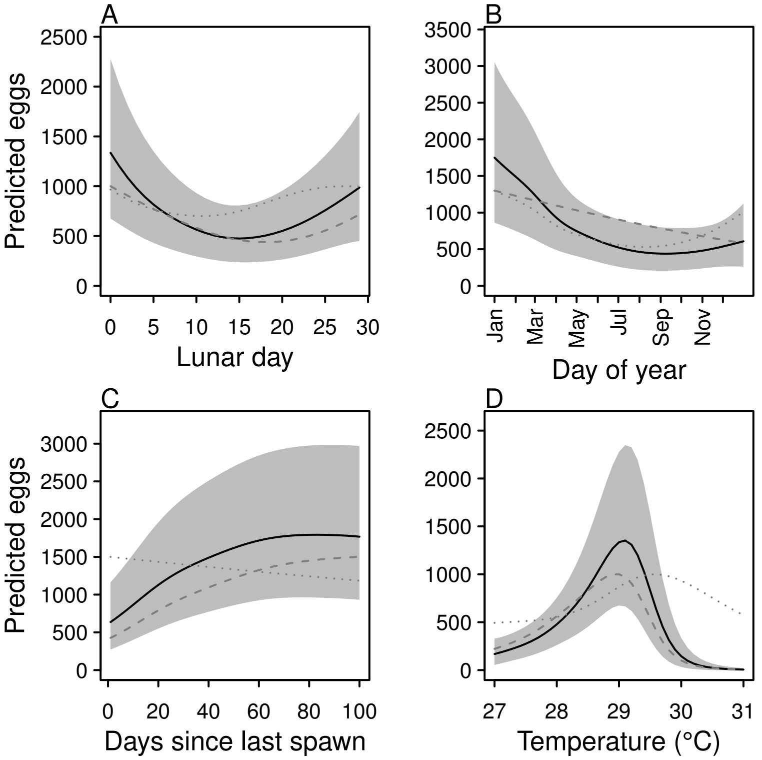
Figure 1. Annual patterns in *Amphiprion clarkii* spawning. (A) Monthly average (±1 standard error) number of spawning events per breeding pair (solid) plotted with daily average temperature (dotted). The numbers across the top indicate the number of pairs observed each month. (B) Monthly average (±1 SE) number of eggs produced per breeding pair (black) and average size of observed clutches (grey). The error bars on clutch size are the standard deviation among clutches within a pair, averaged across pairs.

Figure 2. Spawning patterns over the lunar month. The full moon occurs on lunar day 14, and the new moon on lunar day 29. (A) The average number of spawning events (±SE) per breeding pair by lunar day. (B) The average number of eggs produced per breeding pair for each lunar day.

Figure 3. Fitted terms from the generalized additive mixed effects models (GAMMs) for spawning occurrence (dashed lines), for clutch size (dotted lines), and for the combined prediction of total egg production (solid line with grey shading for standard error). Each subplot corresponds to an explanatory variable in the model, including (A) lunar day, (B) day of year, (C) days since last spawning event, and (D) water temperature. Y-axis is only for the combined prediction of total egg production. Predictions are for a 10 cm female.


A
Spawning events per pair
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Temperature (°C)
27
28
29
30
31
32
7
7
5
5
5
5
7
7
7
7
7
7
B
Egg production per pair
0
1000
2000
3000
4000
Month
January
February
March
April
May
June
July
August
September
October
November
December


A
Spawning events per pair
0.25
0.20
0.15
0.10
0.05
0.00
0
5
10
15
20
25
30
B
Egg Production per pair
2000
1500
1000
500
0
0
5
10
15
20
25
30
Lunar Day


A
Predicted eggs
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
Lunar day
B
3500
3000
2500
2000
1500
1000
500
0
Jan
Mar
May
Jul
Sep
Nov
Day of year
C
Predicted eggs
3000
2500
2000
1500
1000
500
0
0
20
40
60
80
100
Days since last spawn
D
2500
2000
1500
1000
500
0
27
28
29
30
31
Temperature (°C)