Maturation of *Hippocampus guttulatus* and *H. hippocampus* females by manipulation of temperature and photoperiod regimes

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Abstract

The present study provides new and practical information on the maturation of females seahorses *Hippocampus guttulatus* and *H. hippocampus* exposed to different temperature (15 °C constant, 15 – 18 °C or 15 – 21 °C) and photoperiod (10L: 14D - 16L: 8D cycle or 10L: 14D constant). Egg production (Total eggs, clutch size and clutches per female) resulted notably reduced under both short photoperiods and low temperature, especially in *H. guttulatus*. Egg clutches were mainly released with temperatures above 16°C and increasing photoperiods beyond 14L:10D. The highest efficiency under a natural light regime was achieved at 21°C. Biometrics performed in *H. guttulatus* eggs showed that egg volume (V_E) was not affected by temperature level but yolk volume (V_Y) and V_Y/V_E ratio in eggs of females exposed at 15°C were lower than in eggs released at 15-18 °C and 15-21 °C cycles. V_E , V_Y and V_Y/V_E in eggs were not correlated with the photoperiod regimes applied. The present study also provides the first results on shifting of maturation in *H. guttulatus* females submitted to photothermic manipulation of the environment (Treatment D – advanced and drastic change; Treatment A – advanced and accelerated change) or to natural conditions (Treatment N). The response of females to artificial environmental changes was successful and fast. First egg clutches in treatments D and A were released 11 (Treatment D) and 9 (Treatment A) weeks before than in females exposed to natural temperature and photoperiod regimes. However, the best overall results were achieved under natural regimes. In treatment N, total eggs production and average egg clutch size (3,690 and 461 eggs, respectively) were noticeable higher than in treatments D (3,533 and 294 eggs, respectively) and A (150 and 1,809 eggs, respectively). The study demonstrates the feasibility of shifting in female maturation of seahorses and its practical use in the artificial manipulation of the breeding season under captive conditions.

Keywords: Seahorse; *Hippocampus guttulatus*; *Hippocampus hippocampus*; female maturation, temperature, photoperiod, egg.

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1. Introduction

Hippocampus guttulatus and *H. hippocampus* are the only two seahorse species inhabiting the temperate waters of the European coast (Foster and Vincent, 2004). In nature, both species breed over a specific period of the year, from April to October (Boisseau, 1967; Lythgoe and Lythgoe, 1971; Reina-Hervás, 1989; Foster and Vincent, 2004; Curtis and Vincent, 2006). Actions for the development of rearing techniques and conservation of those species have been recently undertaken in Spain (Planas et al., 2009; Olivotto et al., 2011). Even though research on their reproductive biology (Boisseau, 1967; Curtis and Vincent, 2006; Curtis, 2007; Faleiro et al., 2008; Planas et al., 2008a,b, 2010; López et al., 2012; Otero-Ferrer et al., 2012) and rearing techniques has been increasing in the last years, further knowledge is necessary for breeding and broodstock management.

The artificial reproduction of fish requires the establishment of optimal environmental factors and the extension of the breeding season on a year-round basis. Generally, light regime has been considered the main environmental factor of maturation in temperate species of fish (De Vlaming, 1972; Bromage et al., 2001), whereas temperature acts as a secondary factor (Pankhurst and Porter, 2003). Defining optimal temperatures and photoperiod regimes are of pivotal importance in the breeding of whatever species of fish. In seahorses, studies on female maturation are scarce (Lin et al., 2006; Lockyear et al., 2007; Planas et al., 2008b) and optimal environmental conditions for breeding are unknown for most species.

In the artificial propagation of fish under captive conditions, the manipulation of environmental conditions, namely temperature and photoperiod regimes, is a powerful technique for shifting the reproduction season. (Zanuy et al., 1986; Poncin et al., 1987; Bromage et al., 2001; Van der Meeren and Ivannikof, 2006). In seahorses, the artificial shifting of the breeding season has been only documented in the Knysna seahorse *Hippocampus capensis* (Lockyear et al., 2007).

The aim of the present study was: (a) to analyse the effects of different temperature and photoperiod regimes on the maturation of *Hippocampus guttulatus* and *H. hippocampus* females, and (b) to provide the first data on maturation shifting in *H. guttulatus* females by the artificial manipulation of the daylight regime.

2. Material and methods

2.1. Seahorses

Adults of the seahorse *Hippocampus guttulatus* were collected in spring-summer at different sites in the coast of Galicia (NW Spain), whereas seahorses *H. hippocampus* were reared in captivity and provided by ICCM (Telde, Canary Islands). After transportation to the rearing facilities, the fish were tagged with fluorescent tags (VI Alpha Tag, NMT Inc., USA) and maintained for months as described in Planas et al. (2008b). Initially, the fish were acclimatized to 17 - 19 °C and a photoperiod regime of 16L: 8D. Afterwards, temperature was progressively adjusted within the range 15 - 20°C (winter - summer) and controlled by means of a cooling unit and a thermostat ($\pm 0.5^\circ\text{C}$). Natural photoperiods were also applied and the daily light regime adjusted (10L: 14D in December-January - 16L: 8D in June-July).

Average mean weight of seahorses at the onset of the experiments was 24.3 g (19.6 – 28.8 g) in males and 17.2 g (13.1 - 22.9) in females of *H. guttulatus* and 4.1 g (3.1 – 4.6 g) in males and 3.7 g (3 – 3.9) in females of *H. hippocampus*.

Seahorses were fed *ad libitum* twice daily on cultured and enriched adult *Artemia* (length > 5.5 mm; 15-25 days old). *Artemia* on-growing was carried out using mixtures of *Isochrysis galbana*, lyophilised *Spirulina* and Prolon (Inve, Spain) (Quintas et al., 2007). Adult *Artemia* was enriched on mixtures of *I. galbana*, Prolon and ACE (*Artemia* condition enhancer; Inve), as described in Planas et al. (2008b).

2.2. Experiment 1: Effects of temperature and photoperiod regimes in *H. guttulatus* and *H. hippocampus*.

Late in January 2008, a total of 32 *H. guttulatus* and 12 *H. hippocampus* adults were randomly distributed in seven 165 l aquaria: 4 aquaria for *H. guttulatus* (4♀+ 4♂ per aquarium) and 3 aquaria for *H. hippocampus* (2♀+ 2♂ per aquarium). Temperature and photoperiod conditions at the onset of the experiment were 15 °C and 10L: 14D, respectively. Subsequently, the aquaria were exposed to 4 treatments (A, B, C and D) with different temperature and photoperiod regimes as shown in Table 1. Photoperiod and temperature combinations were

imposed by the limited amount of seahorses and aquaria available. The experiment was maintained for 12 months.

2.3. Experiment 2: Induced seasonal shifting of maturation in *H. guttulatus*.

The experiment was initiated in early December 2009 and carried out only on *H. guttulatus* due to the unavailability of *H. hippocampus* adults. Twenty eight males and thirty two females were distributed with sex separated in six 165 l aquaria and maintained for six months.

The following photoperiod treatments were applied:

- Treatment A: Advanced change (12♀ and 10♂). The photoperiod regime was compressed and advanced 3 months from 10L:14D (early December) to 16L:8D (from late February). The temperature was progressively raised (1°C/two weeks) from 15 °C (early December) to 20°C (from mid February).
- Treatment D: Drastic change (12♀ and 10♂). The photoperiod cycle was swiftly changed in early December from 10L:14D to 16L:8D and the temperature was progressively raised (1°C/week) from 15 °C (early December) to 20°C (from early January)
- Treatment N: Natural (8♀ and 8♂). A natural photoperiod was applied (from 10L: 14D in December-January to 16L: 8D in June-July). The temperature was progressively adjusted from 15°C (December-March) to 20°C (June-September).

For each treatment, one receptive male (colour changing) was weekly transferred to the corresponding female's aquaria. Five days later, transferred males were reintroduced into their original aquaria. This procedure was repeated weekly for 6 months, using all available seahorses.

In both experiments, egg clutches dropped by females were collected with care by siphoning and transferred to a Petri dish for egg counting. Digital photographs of eggs were taken for biometrics. Eggs were generally released during the day and counted immediately after release. Exceptionally, eggs were released at night and collected early in the morning.

Newborns were computed as eggs and the time of eggs release (eggs transfer from female to males) was recalculated taking into account the duration of the interbrood interval (Planas et al., 2008a).

2.4. Data and statistical analyses

In Experiment 1, egg volume and yolk volumes were calculated for *H. guttulatus* as described by Planas et al. (2010). Measurements were made from digital photographs.

Statistical analyses and calculations were performed using the software package Statistica 8.0 (StatSoft). Mean clutch sizes and egg biometrics were compared by applying ANOVA. Unbalanced ANOVA was used in Experiment 1 (treatment nested in species) as treatment A was lacking in *H. hippocampus*. Differences among treatments were tested using post-hoc nonparametric Duncan's test. A significance level of 0.05 was used in statistical tests.

3. Results

3.1. Experiment 1

Considering all treatments, most egg clutches were collected from mid-May to October, both in *H. guttulatus* and *H. hippocampus*, though some clutches were also released in April and December (Fig. 1).

A summary on the effect of photothermic manipulation in egg production is provided in Table 2. The number of clutches produced by female was lower in *H. guttulatus* (0.7-3.5) than in *H. hippocampus* (4.0-6.0). Under natural regime (Treatments B and D), clutch size and maximum clutch size in *H. guttulatus* (282 ± 99 and 418, respectively) was higher than in *H. hippocampus* (135 ± 104 and 359, respectively), but the total number of eggs produced per female was similar in both species. Female maturation was influenced by temperature levels, though essentially controlled by light regime. A constant winter light regime (Treatment C) conducted to a generalised drop in all maturation parameters analysed, especially in *H. guttulatus*. Female maturation was enhanced by increasing temperature levels. Huge increases were recorded at 21°C, in total number of eggs produced per female and, to a lesser extent, in maximum clutch size. However, average clutch size at 18 and 21°C (Treatments B and D) were similar in both species and higher than at 15 °C.

Biometrics performed in eggs of *H. guttulatus* (Table 3) showed that egg volume (V_E) was not affected by photothermic regimes. Conversely, eggs from females maintained at a constant temperature of 15°C and natural photoperiod regime (Treatment A) had lower yolk volume (V_Y) and V_Y/V_E ratios than eggs from other treatments (Table 4). V_E , V_Y and V_Y/V_E in eggs of females submitted to different photoperiod regimes at 18 °C (Treatments B and C) were not significantly different.

3.2. Experiment 2

Modifications of the natural photoperiod regime conducted to an advancement in the maturation of *H. guttulatus* females (Figure 2). The first egg clutches in females submitted to artificial changes in temperature and photoperiod regimes were released 35 (early January in Treatment D) and 48 days (mid January in Treatment A) after the start of the experiment. The first clutch release in treatment N was recorded 122 days after the onset of experimentation (late May). The environmental conditions at first egg release were 13L:11D and 18°C in Treatment A, 16L:8D and 20°C in Treatment D and 15L:9D and 18 °C in Treatment N.

Average clutch size in females submitted to photoperiod manipulation (201 ± 160 and 208 ± 159 eggs in Treatments A and D, respectively) was lower but not statistically significant than in Treatment N (263 ± 137 eggs) (Table 4). The overall egg production (total eggs and eggs per female) in treatment N (3690 and 461, respectively) was higher than in treatments D (3533 and 294, respectively) and especially A (1809 and 150). The number of egg clutches in treatments N and D (1.75 and 1.42, respectively) were higher than in treatment A (0.75).

4. Discussion

One of the first steps in the artificial propagation of fish is the identification of the most favourable environment required for a species to undergo reproductive maturation. Very little information is known on the optimal conditions for seahorse reproduction. Seahorse females are batch spawners with repeated mates within a breeding season and egg production synchronised with brooding in males (Foster and Vincent, 2004; Curtis, 2007; Olivotto et al., 2008; Planas et al., 2010). Following spawning and the transfer of mature eggs to males's brood pouch, a new clutch of eggs will be released within a period of time after the development of a new cohort of follicles. Inter-clutch interval in *H. guttulatus* is highly dependent on temperature (46 - 27,5 days at 16 - 20 °C, respectively) (Planas et al., 2010). In nature, the breeding season for *H. guttulatus* extends from March to September-November depending on latitude (Boisseau, 1967; Reina-Hervás, 1989; Curtis, 2007), with a peak in June - August (Curtis and Vincent, 2006). Spring-Summer temperatures are optimal for the growth and survival of newborn (Planas et al., 2012). In captive conditions, *H. guttulatus* females submitted to natural photoperiod and temperature regimes were able to successfully mature over eight months, from March (16-17 °C; 13L:11D) to October (18 °C; 13L:11D), with a period of maximal

eggs release from June to August (16L:8D - 14L:10D; 18-20°C) (Planas et al., 2008b, 2010). Knowledge on the breeding of *H. hippocampus* is far less documented. It has been reported that the species breed from Spring (April) to Autumn (October) (Foster and Vincent, 2004), that successful breeding occurs with temperatures of 22-23 °C and a light regime of 10L:14D (Otero-Ferrer et al., 2012) and that the gestation duration is 21-32 days (Foster and Vincent, 2004).

In Experiment 1, the effects of various levels of temperature and daily light regimes on the maturation of females in the temperate water species *Hippocampus guttulatus* and *H. hippocampus* were analysed. The results agree with previous data on the breeding season of *H. guttulatus* and *H. hippocampus* in the wild and in the laboratory. First egg clutches in *H. guttulatus* were released in May, with 17-18 °C and 16L:8D. Considering that the inter-clutch interval in this species is 35.5 days at 18 °C (Planas et al., 2010), the first response of female gonads to changes in environmental conditions would occur when the photoperiod increased up to 14L:10D with temperatures of 15.5-16 °C. High temperatures (18 - 21 °C) did not seem to play a pivotal role in the onset of female maturation but clearly enhanced maturation performance (total clutches, total egg production and maximal clutch size) throughout the breeding season. Similar findings were observed in *H. hippocampus*, though the extension of the maturation period was wider than in *H. guttulatus*. In seahorses, the effect of light regime has been only studied in *H. capensis* (Lockyear et al., 1997), in which several combinations of photoperiod and high temperatures were successful in extending the breeding season. The effect of temperature on gonad development (gonadosomatic index) and reproductive efficiency (fecundity and spawning) in *H. white* and *H. kuda* has been reported earlier (Wong and Benzie, 2003; Lin et al., 2006). According to our results, it is likely that female maturation in *H. guttulatus* and *H. hippocampus* species be mainly controlled by photoperiod with an additive effect of increasing temperature levels. Additive effects of environmental factors have been reported on ovulation of Atlantic salmon (*Salmo salar*) (King and Pankhurst, 2007).

A constant winter temperature combined with a spring-summer photoperiod or a constant winter photoperiod combined with a spring-summer temperature did not inhibit egg production but reduced drastically maturation performance by limiting the number of egg clutches, the clutch size and the total number of eggs produced. The overall performance of egg production was also decreased by low temperatures due very likely to an increase of the inter-clutch interval and consequently of the total number of clutches during the whole breeding season

(Planas et al., 2010). Among seahorses, Lockyear et al. (1997) pointed out that *H. capensis* reproduced equally well regardless of the photothermic regimes applied. However, temperatures below the average temperature level occurring in the natural habitat of *H. capensis* were not considered in that study. The ability of females to spawn successfully under constant photoperiod regimes has been reported in other fish species (e.g. *Salmo gairdneri*, *Dicentrarchus labrax*), indicating that changes in photoperiod would not be essential for maturation and that photoperiod serves to entrain an endogenous rhythm of maturation rather than having a direct driving influence on reproduction of the rainbow trout (Duston and Bromage, 1987; Carrillo et al., 1993). The existence of endogenous annual reproductive rhythms in seahorses deserves further investigation.

Egg quality can be defined as the ability of the egg to be fertilized and subsequently develop into a normal embryo. Egg size or yolk content are parameters commonly used to assess egg quality in fish. Egg quality can be affected by many environmental (photoperiod, temperature, salinity and pH of the water) and biological factors acting at various steps of the oogenetic process (Brooks et al., 1997). For example, the control of ovulation in the rainbow trout *Oncorhynchus mykiss* by both hormonal induction and photoperiod manipulation induced significant changes in the egg mRNA abundance of specific genes and that the egg mRNA abundance of prohibitin 2 (PHB2) was negatively correlated with the developmental potential of the egg (Bonnet et al., 2007). Water temperature during spawning and embryogenesis is particularly important in affecting egg quality as it may affect metabolism, activity and structure of the developing embryo (Brooks et al., 1997). The effect of temperature on gonad development and detrimental effects of low temperatures have been reported in *H. kuda* (Lin et al., 2006). Seahorse eggs are large, heavy, non-buoyant and contain numerous small lipid droplets. In *H. guttulatus*, the average size of eggs is equivalent to a sphere of 1.7 mm in diameter, with yolk accounting for about $59.0 \pm 9.5\%$ total egg volume (Planas et al., 2010). The lower relative yolk content (V_Y/V_E) in eggs released at low temperatures suggests an inferior quality than those matured at 18-21 °C. This finding could be related to a lower feeding activity of adults at low temperatures and the resultant decrease in the availability and mobilization of nutrients. Effects of different photoperiod/temperature combinations on size and weight of newborn have been reported earlier in the seahorse *H. capensis* (Lockyear et al., 1997).

The extension of the breeding season in fish to allow juveniles production on a year-round basis can be artificially controlled by manipulation of environmental factors, such as photoperiod or water temperature, or by hormonal induction (Poncin et al., 1987; Zohar, 1989; Van der Meeren and Ivannikov, 2006; Mylonas et al., 2010). The present study provides the first results in females of *H. guttulatus* on maturation shifting and on extension of egg production by the application of advanced photoperiod regimes, drastic or progressive, combined with the corresponding temperature changes. Similarly to *H. capensis* (Lockyear et al., 1997), female maturation in winter was successful and the response of gonads to changes was fast, with advances of 11 (drastic change) and 9 (advanced change) weeks with respect to natural photothermic regimes.

In conclusion, female maturation in captive seahorses *H. guttulatus* and *H. hippocampus* was especially dependent on light regime but boosted by increasing temperature levels. Egg clutches were mainly released with temperatures above 16°C and increasing photoperiods beyond 14L:10D. The highest efficiency under a natural light regime was achieved at 21°C. Shifting in maturation of *H. guttulatus* females was successful. Some experiments have been undertaken recently with two groups of adult seahorses exposed to a drastic change as in treatment D in the present study. Several batches of newborn have been released since 1.5 months after the start of the experiment. The size and the general status of the juveniles were excellent. Newborn are being cultivated under different experimental conditions and the survivals achieved at the present are very high (>80% at different ages up to 1 month). These preliminary results demonstrate both the practical and successful application of the results achieved in present study and the high-quality of newborn. Anyway, further research is needed to verify the existence of endogenous reproductive rhythms or potential long-term effects in subsequent breeding seasons and physiological alterations (Van der Kraak and Pankhurst, 1996; Webb et al., 2001; Van der Meeren and Ivannikov, 2006).

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Fig.1. Experiment 1: Number of egg clutches and clutch size (number of eggs per clutch) released by females of *H. guttulatus* and *H. hippocampus* exposed to different temperature and photoperiod regimes (Treatments A, B, C and D, and pooled data).

Fig.2. Experiment 2: Number of egg clutches and clutch size (number of eggs per clutch) released by *H. guttulatus* females submitted for six months to natural (Natural) or artificially (Advanced or Drastic) manipulated photoperiod regimes. The delay in first clutch release since the start of the experiment and the period of egg release are shown (Horizontal bars).

FIGURE 1

FIGURE 2

Table 1 – Experiment 1: Temperature and photoperiod regimes applied to females of *Hippocampus guttulatus* and *H. hippocampus*. Photoperiod regime: Seahorses were exposed to a progressive change of light regime from 10L: 14D in January to 16L: 8D in June-July (10L:14D - 16L:8D cycle) or to a constant light regime of 10L:14D (10L:14D constant). Temperature regime: Seahorses were exposed to constant temperature (15°C constant) or to progressive changes of temperature from 15 °C in December-April to 18 or 21 °C in June-September (15 – 18 °C cycle or 15 – 21 °C cycle, respectively).

Photoperiod regime	Temperature regime		
	15°C constant	15 – 18 °C cycle	15 – 21 °C cycle
10L:14D - 16L:8D cycle	Treatment A <i>H. guttulatus</i>	Treatment B <i>H. guttulatus</i> <i>H. hippocampus</i>	Treatment D <i>H. guttulatus</i> <i>H. hippocampus</i>
10L:14D constant	-	Treatment C <i>H. guttulatus</i> <i>H. hippocampus</i>	-

Table 2 – Experiment 1: Effect of the temperature level on the egg production of *Hippocampus guttulatus* and *H. hippocampus* females (egg production) exposed to different photoperiod regimes (Treatments A, B, C and D). Photoperiod regime: Seahorses were exposed to a progressive change of light regime from 10L: 14D in January to 16L: 8D in June-July (10L:14D - 16L:8D cycle) or to a constant light regime of 10L:14D (10L:14D constant). Temperature regime: Seahorses were exposed to constant temperature (15°C) or to progressive changes of temperature from 15 °C in December-April to 18 or 21 °C in June-September (15 – 18 °C cycle or 15 – 21 °C cycle, respectively). Different letters between treatments for clutch size indicate significant differences (post-hoc Duncan's Test, $P < 0.05$) (ANOVA, $P < 0.0001$).

Species	Treatment	Temp (°C)	Photoperiod	Clutches collected	Clutche s/ Female	Total Eggs	Eggs/Clutch (mean ± sd)	Max Clutch Size	Eggs/ Female
<i>Hippocampus guttulatus</i>	A	15	10L:14D - 16L:8D	8	2.0	540	68 ± 39 ^a	119	135
	B	15-18	10L:14D - 16L:8D	8	2.0	2253	282 ± 99 ^b	418	563
	C	15-18	10L:14D constant	3	0.7	199	66 ± 42 ^a	115	50
	D	15-21	10L:14D - 16L:8D	14	3.5	3861	276 ± 135 ^b	486	965
<i>Hippocampus hippocampus</i>	B	15-18	10L:14D - 16L:8D	8	4.0	1067	133 ± 73 ^a	271	534
	C	15-18	10L:14D constant	8	4.0	688	86 ± 55 ^a	181	344
	D	15-21	10L:14D - 16L:8D	12	6.0	1625	135 ± 104 ^a	359	813

Table 3 – Experiment 1: Effect of temperature and photoperiod regimes (Treatments A, B, C and D) on egg volume (V_E), yolk volume (V_Y) and V_Y/V_E ratio production in females of *H. guttulatus*. Photoperiod regime: Seahorses were exposed to a progressive change of light regime from 10L: 14D in January to 16L: 8D in June-July (10L:14D - 16L:8D cycle) or to a constant light regime of 10L:14D (10L:14D constant). Temperature regime: Seahorses were exposed to constant temperature (15°C) or to progressive changes of temperature from 15 °C in December-April to 18 or 21 °C in June-September (15 – 18 °C cycle or 15 – 21 °C cycle, respectively). Different letters between treatments within a column indicate significant differences (post-hoc Duncan's Test; $P < 0.05$) (ANOVA, $P = 0.221$ for V_E , $P < 0.001$ for V_E and $P = 0.021$ for V_Y / V_E).

Treatment	Photoperiod	Temp (°C)	n	V_E (μl)	V_Y (μl)	V_Y / V_E
A	10L:14D - 16L:8D	15	118	2.176 ± 0.639 ^a	1.227 ± 0.282 ^a	0.593 ± 0.153 ^a
B	10L:14D - 16L:8D	15-18	120	2.205 ± 0.415 ^a	1.423 ± 0.419 ^b	0.657 ± 0.180 ^b
C	10L:14D constant	15-18	60	2.252 ± 0.480 ^a	1.372 ± 0.324 ^b	0.622 ± 0.135 ^b
D	10L:14D - 16L:8D	15-21	120	2.324 ± 0.690 ^a	1.415 ± 0.368 ^b	0.638 ± 0.163 ^b

Table 4 – Experiment 2: Effect of photoperiod manipulation in Treatments A, D and N (Advanced change, drastic change and natural photoperiod, respectively) on egg production of *H. guttulatus* females. Temperature regime: 15–18°C cycle. Different letters between treatments for clutch size indicate significant differences (post-hoc Duncan's Test; $P < 0.05$) (ANOVA, $P = 0.517$).

Photoperiod regime	Clutches collected	Clutches/Female	Total Eggs	Eggs/Clutch (mean $\pm$ sd)	Max Clutch Size	Eggs/Female	First Clutch Release (Days)
Advanced change	9	0.75	1809	201 $\pm$ 160 ^a	509	150	48
Drastic change	17	1.42	3533	208 $\pm$ 159 ^a	539	294	35
Natural	14	1.75	3690	264 $\pm$ 137 ^a	486	461	122