AN ABSTRACT OF THE THESIS OF

Kitty Sue (Hines) Bobbitt for the Master of Science

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Title: Steroid Levels and Gravid Coloration in Crotaphytus collaris

Abstract approved: Robert A. Clarke

Progesterone and testosterone were measured by radioimmunoassay in serum samples taken from female Crotaphytus collaris. Data indicate that progesterone levels are higher than testosterone in gravid colored females, even though levels for both hormones were unexpectedly low. In specimens exhibiting orange-red coloration, the serum concentrations of testosterone were 0.046 ng/ml and 0.014 ng/ml. Other testosterone concentrations were low and unreliable. Other specimens with orange-red coloration had a mean serum concentration of progesterone of 2.07 ng/ml $\pm$ 0.75. It was hypothesized that increases and decreases of gravid color intensity would accompany increases and decreases in the steroid levels. Gravid coloration was analyzed and then compared to the steroid levels. Similar patterns between gravid color intensities and testosterone levels were evident in two specimens. However, no final conclusion can be drawn because of inconclusive data. Data comparing gravid coloration and progesterone levels were also inconclusive.

STEROID LEVELS AND GRAVID COLORATION IN *CROTAPHYTUS COLLARIS*

A Thesis
Submitted to
the Division of Biological Sciences
Emporia State University
Emporia, Kansas

In Partial Fulfillment
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Master of Science

by
Kitty Sue (Hines) Bobbitt
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Approved for Major Department

Approved for Graduate Council

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INTRODUCTION

Most of the studies on lizard color changes have been done on the chromatophores' response to environmental stimuli such as: temperature, light, and background color (Smith 1946; Parker 1948). These pigment-containing cells are under primary control by melanophore-stimulating hormone (MSH) secreted by the pars intermedia (Gorbman et al. 1983). The color change responses serve the following functions: 1) protecting the animal from predators by cryptic or warning coloration, 2) protecting body tissues against excessive radiation, and 3) signaling a state of sexual readiness (Gorbman et al. 1983; Porter 1972; Turner and Bagnara 1971). Color change ability is restricted to the reptilian families of chamaeleons, geckos, xantusiids, agamids, and the iguanids (Smith 1946).

In addition to color changes made in response to environmental stimuli, some female iguanid lizards respond with color changes to ovarian hormone stimulation during reproductive stages. In Crotaphytus collaris the orange-red coloration is dependent upon the gravid state (Fitch 1965). The spots appear after copulation and usually regress after egg-laying (Cooper and Ferguson 1972). In other iguanid lizards, the orange colors appear when eggs are mature in the ovary and before copulation (Vinegar 1972).

The following observations were made that furthered research in this area. Fitch (1956) observed a postnuptial color change in gravid female collared lizards. This color change was noted by the appearance of orange-red lateral spots approximately three to four days after

copulation. The bright orange-red spots appearing after copulation were also reported by Cooper and Ferguson (1972).

Other authors have described gravid female color in various iguanid species. This coloration of female Holbrookia propinqua consists primarily of orange along the sides and on the ventral tail surface (Cooper and Clarke 1982). Medica et al. (1973) noted that Crotaphytus wislizenii develops red-orange coloration on the sides of the face, body, and ventral tail surface. Vinegar (1972) observed the surrounding or replacement of blue throat patches by orange color in breeding Sceloporus virgatus.

Studies done on Crotaphytus collaris and other lizards showing postnuptial color changes report that steroid hormones are able to induce these changes. Cooper and Ferguson (1972) reported that injections of progesterone and testosterone produce the color changes found in gravid Crotaphytus collaris. They also found that estrogen injections by themselves do not induce the orange color but may act as a primer for progesterone (Cooper and Ferguson 1973). These same results were found by Medica et al. (1973) in Crotaphytus wislizenii and by Cooper and Clarke (1982) in Holbrookia propinqua. Arslan et al. (1978) found that progesterone, testosterone, and estradiol plasma concentrations are elevated during the gravid phase of Uromastix hardwicki.

The goals of this research were to determine if blood serum levels of progesterone and testosterone increase during the gravid phase of the iguanid lizard Crotaphytus collaris and to determine how the steroid hormone levels compared with each other. A comparison was also

made between gravid phase color levels and hormone levels to determine if any correlations existed.

MATERIALS AND METHODS

Crotaphytus collaris specimens were collected from May through July, 1984. Two collection sites were most used: Highway 160 in Montgomery County, Kansas, at the old rock quarry opposite the Elk City Dam and Reservoir, and two miles southwest of the county line between Lyon and Chase counties in Chase County, Kansas. Few specimens were obtained in May due to unusually cold and rainy weather. Serum samples from the lizards caught in May were used to learn assay techniques.

Captured lizards were taken to Ross Natural History Reservation where they were toe clipped for identification and released in three ten-feet by five-feet galvanized sheet metal outdoor cages. The cages were partitioned into six living areas. Large rocks were arranged to simulate natural habitat for sunning and shelter. Each living unit contained no fewer than two female lizards and one male lizard at any time. From October through November, 1984, the lizards were housed in indoor cages. The lizards were maintained on a diet of crickets, supplemented by mealworms and grasshoppers. Lizards that failed to reach a weight of 15.0 g were not used in the study.

Weekly data were collected on the lizards. Specimens were weighed and examined externally to check for adverse effects from the study. Blood samples were collected by cardiac puncture using Plastipak disposable 25 G 5/8" needles on 1 cc insulin syringes. About 0.5 ml blood was drawn from each lizard. A Doppler was used in the beginning of the study to increase proficiency of the technique. To minimize damage to the lizards, if no blood was collected after three attempts

in one day, the lizard was returned to the cage and further attempts at collection were suspended until the following week. Blood serum was separated by centrifugation and frozen at 0 C for later use.

Gravid orange-red coloration was evaluated weekly and any changes recorded using Munsell Color Chips (available from Munsell Color Co., 2441 N. Calvert Street, Baltimore, Maryland 21218). Colors were ranked by number with the increase in numbers indicating a shift toward the red end of the color spectrum (Table 1).

Table 1. Ranking of Munsell Color Chips

COLOR CHIP	RANKING
No gravid color	0
5Y 9/6	1
10YR 9/4	2
5YR 8/6	3
10YR 8/8	4
10YR 8/14	5
5YR 7/10	6
5YR 7/12	7
5YR 7/14	8
5YR Max	9
10R 6/12	10
10R 5/10	11
10R 5/14	12
10R 5/16	13

Radioimmunoassays (RIA) were conducted on the samples to ascertain serum concentrations of testosterone and progesterone. Materials and methods for the assays follow the progesterone and testosterone steroid hormone test kits designed by Wein Laboratories, Inc., P.O. Box 227, Succasunna, New Jersey 07876 (Appendices A and B). Four alterations were made in the procedures. In the progesterone procedure, 1.0 ml of distilled water was added to each tube instead of 2.0 ml. Five milliliters of petroleum ether were added instead of 20 ml. In the testosterone procedure, 5.0 ml to 7.0 ml aliquots were transferred into tubes after methylene chloride was added instead of the 4.0 ml recommended. After shaking the methylene chloride mixture, the mixture was centrifuged and the upper aqueous layer aspirated off.

After drying the testosterone samples, a one millimeter thick white ring was located at the bottom of each sample tube. The tubes were incubated for an extra twenty minutes after adding the 3H-Testosterone solution and the phosphate buffer to see if the ring would resuspend into the solution. The ring was thought to be a layer of insoluble lipids and proteins (Parrish 1985). It did not resuspend.

Other methods were then used to attempt resuspension of the layer to allow more accurate detection of hormone levels. These methods included adding different substances to the samples to dissolve the layer. The substances added include: 1) 100 ul of Triton X-100 detergent, 2) one ml Gel EDTA buffer, 3) 0.5 ml four percent bovine serum albumin in phosphate buffer, and 4) 100 ul alcohol (Smalley 1985). The alcohol addition did help and resulted in a cloudy solution without a ring. The alcohol addition did not appear to denature the

RIA antibody.

Serum from lizards # 30, # 17, # 24, and # 33 was tested for testosterone levels. The samples were not treated with alcohol. Serum from lizards # 22 and # 8 were also tested for testosterone concentration. These samples were treated with alcohol. The testosterone study could not be repeated due to lizard serum inavailability.

Blood serum from successive draws of one lizard were pooled to provide a minimum of 500 ul of test serum for the RIAs. All assays were done in duplicate. Unknown serum concentrations were derived from the standard curves set up. Because assays were done only in duplicate, interassay variability is not available. Coefficients of variation could not be calculated for the testosterone study. Data were not available to calculate a standard deviation. The coefficients of variation for the progesterone study are as follows: lizard # 28 - 75 %, lizard # 34 - 72 %, lizard # 32 - 59 %, lizard # 23 - 58 %, lizard #26 - 52 %, lizard #12 - 80 %, lizard # 60 - 150 %, and lizard #31 - 105 %. The binding sensitivity to progesterone was 25 %. The binding sensitivity of the assays to testosterone was 29 %.

RESULTS AND DISCUSSION

Duration of Gravid Color

The bright orange-red coloration indicative of the gravid condition is more easily recognized in contrast to the regular tan to light brown body color of female Crotaphytus collaris. This fact and the knowledge that most of the study lizards were captured in June help explain why 13 of the 22 females captured already exhibited gravid color spots (Fig. 1 through Fig. 13). According to Ferguson's (1976) stages of reproductive cycling, lizards in vitellogenic stages exhibit orange gravid colors. It was determined that most of the lizards captured were already in either Stage II, late vitellogenic, or Stage III, preovulatory.

Only one female did not exhibit orange-red coloration at the time of capture; neither did that condition change throughout the study (Fig. 14). Although specimen # 12 did have orange-red spots when captured in May, these colors were completely faded by July 1 (see Fig. 7). The orange-red spots of specimen # 8 were already fading when captured and were completely gone by June 24 (see Fig. 1). These three lizards were used as controls of the study. Because no lizards became gravid after capture, no measurements could be made on the appearance of the orange-red spots and their change to brighter intensities. Hormone levels that correspond to appearing gravid colors also could not be measured.

All other specimens did not lose their orange-red markings until late summer to early fall. November 25 is the latest recorded date of the orange coloration during this study. This may be the result of

Figure 1. The relationship between gravid coloration vs time for C. collaris # 8 (control).

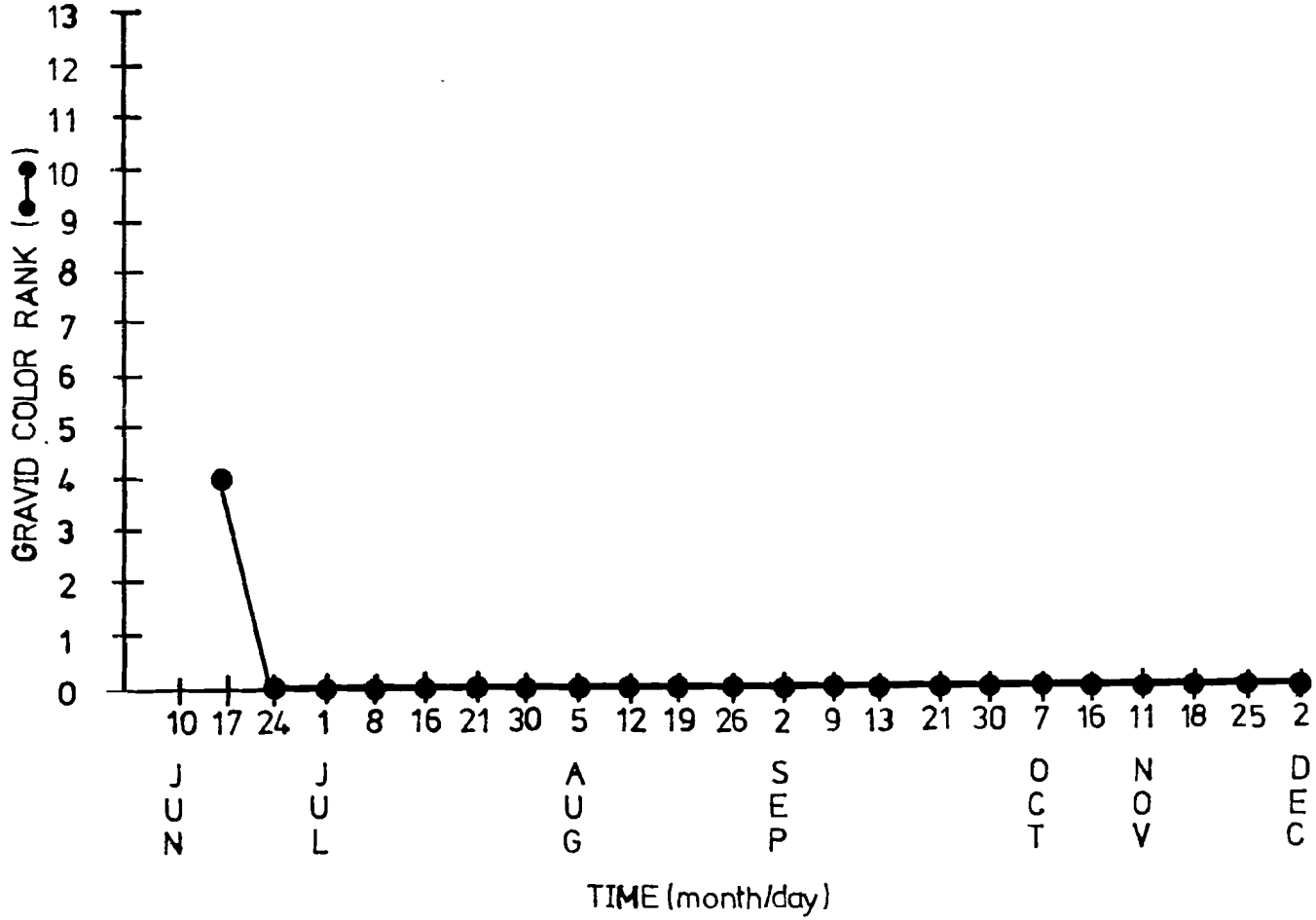

GRAVID COLOR RANK (—●—)
13
12
11
10
9
8
7
6
5
4
3
2
1
0
10 17 24 1 8 16 21 30 5 12 19 26 2 9 13 21 30 7 16 11 18 25 2
JUN
JUL
AUG
SEP
OCT
NOV
DEC
TIME (month/day)

Figure 2. The relationship between gravid coloration vs time for *C. collaris* # 17.

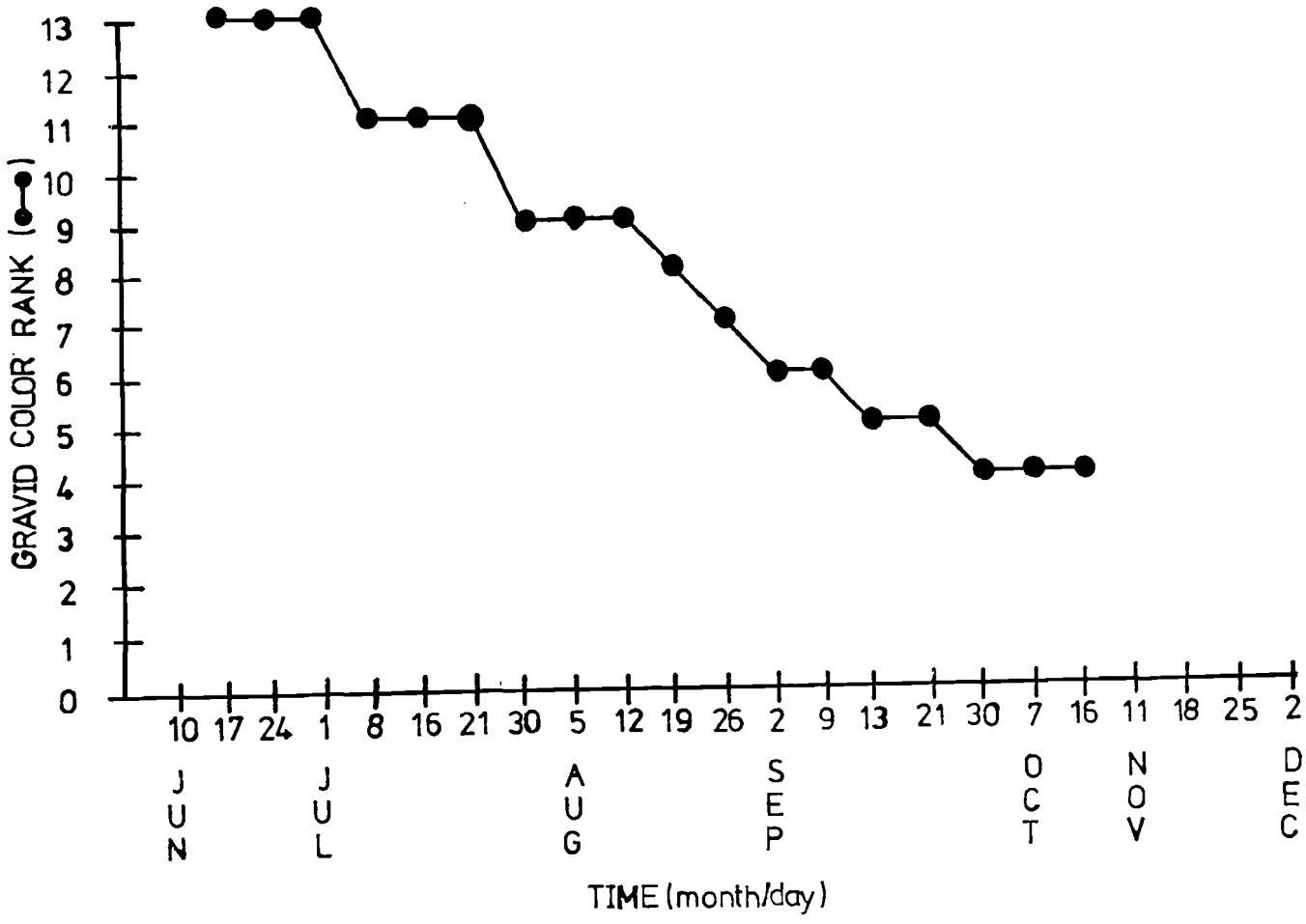
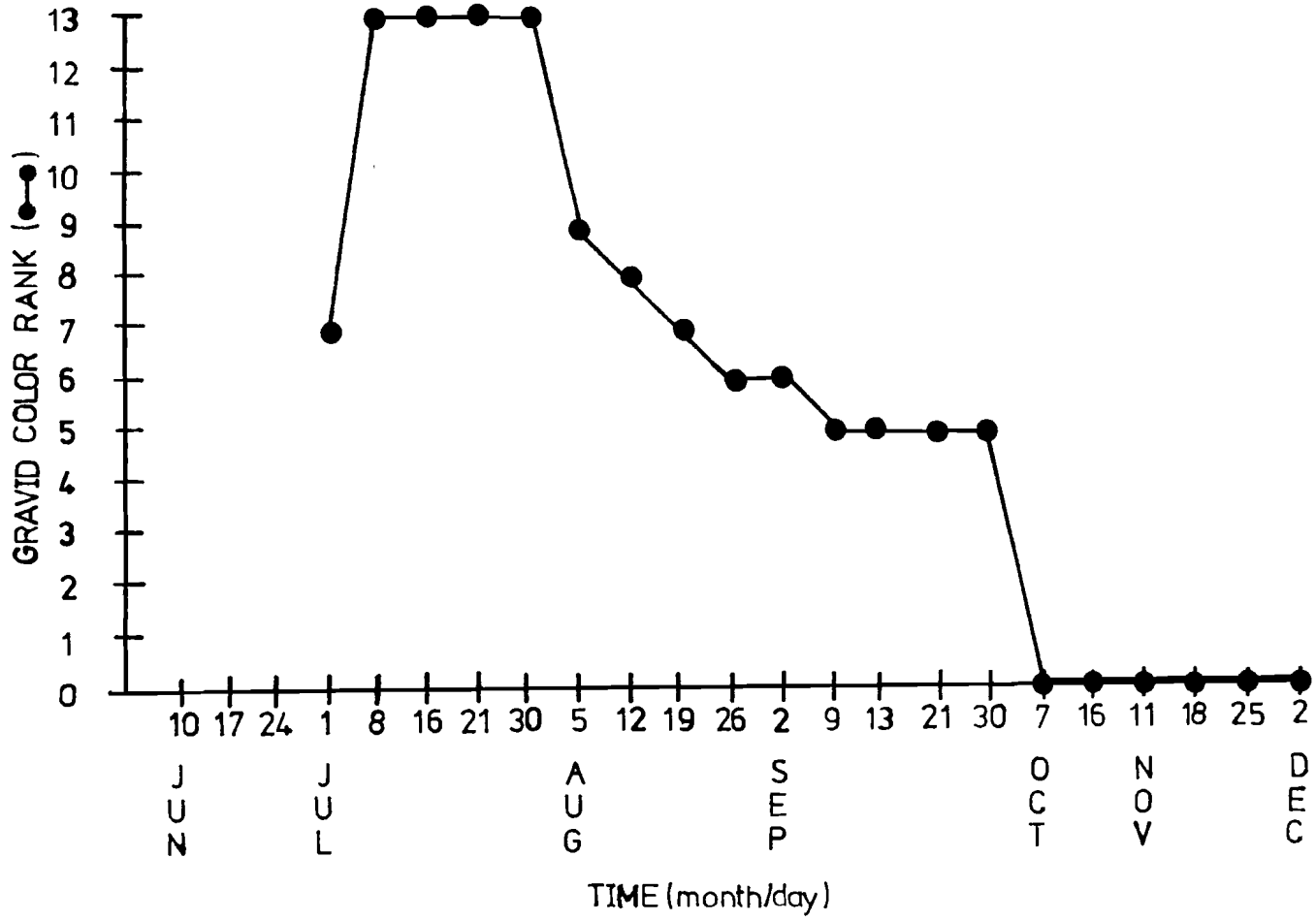

GRAVID COLOR RANK (●—●)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
10 17 24 1 8 16 21 30 5 12 19 26 2 9 13 21 30 7 16 11 18 25 2
JUN
JUL
AUG
SEP
OCT
NOV
DEC
TIME (month/day)

Figure 3. The relationship between gravid coloration vs time for C. collaris # 22.


GRAVID COLOR RANK (●)
13
12
11
10
9
8
7
6
5
4
3
2
1
0
10 17 24 1 8 16 21 30 5 12 19 26 2 9 13 21 30 7 16 11 18 25 2
JUN
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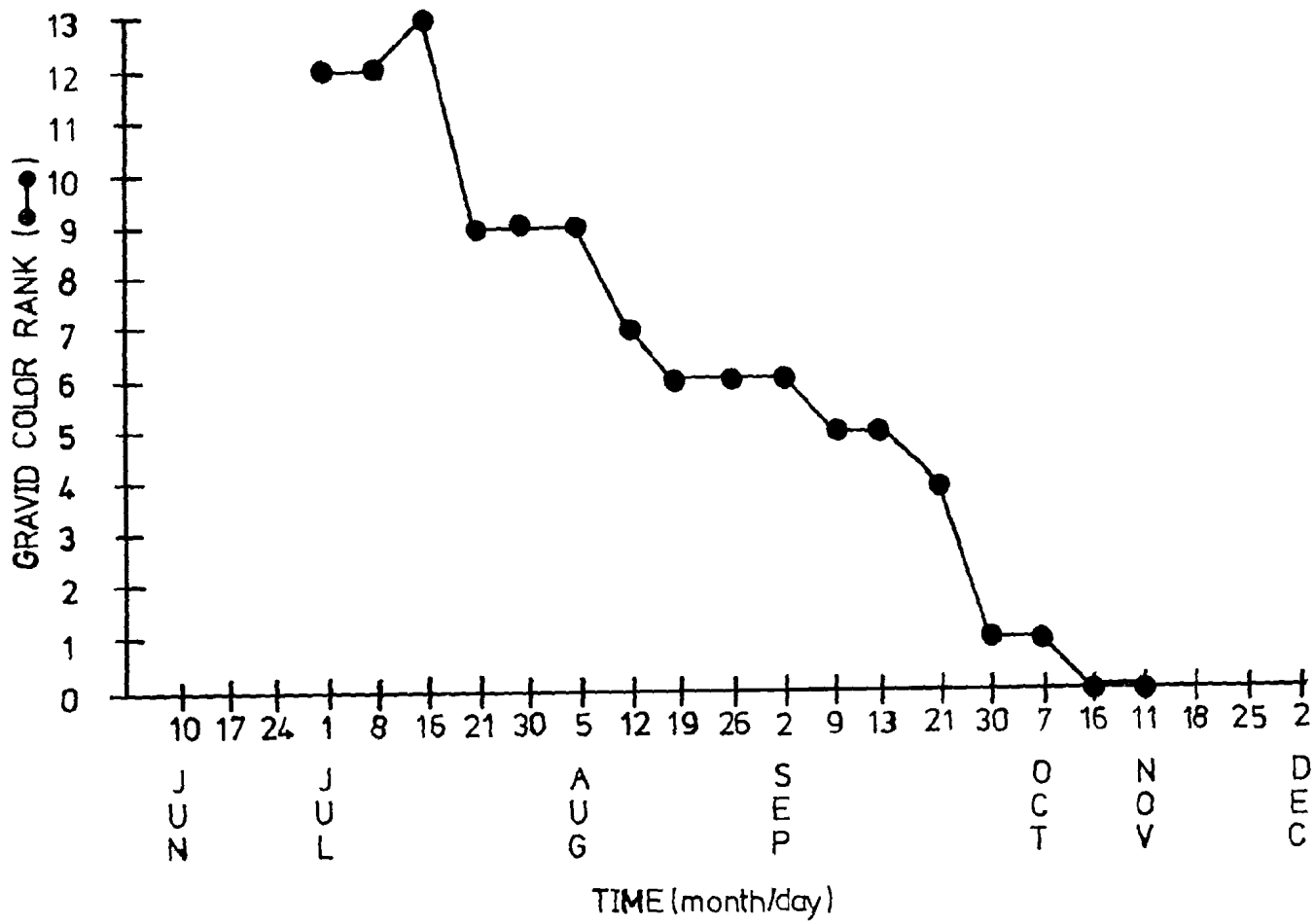
SEP
OCT
NOV
DEC
TIME (month/day)

Figure 4. The relationship between gravid coloration vs time for C. collaris # 30.


GRAVID COLOR RANK (●)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
10 17 24 1 8 16 21 30 5 12 19 26 2 9 13 21 30 7 16 11 18 25 2
JUN
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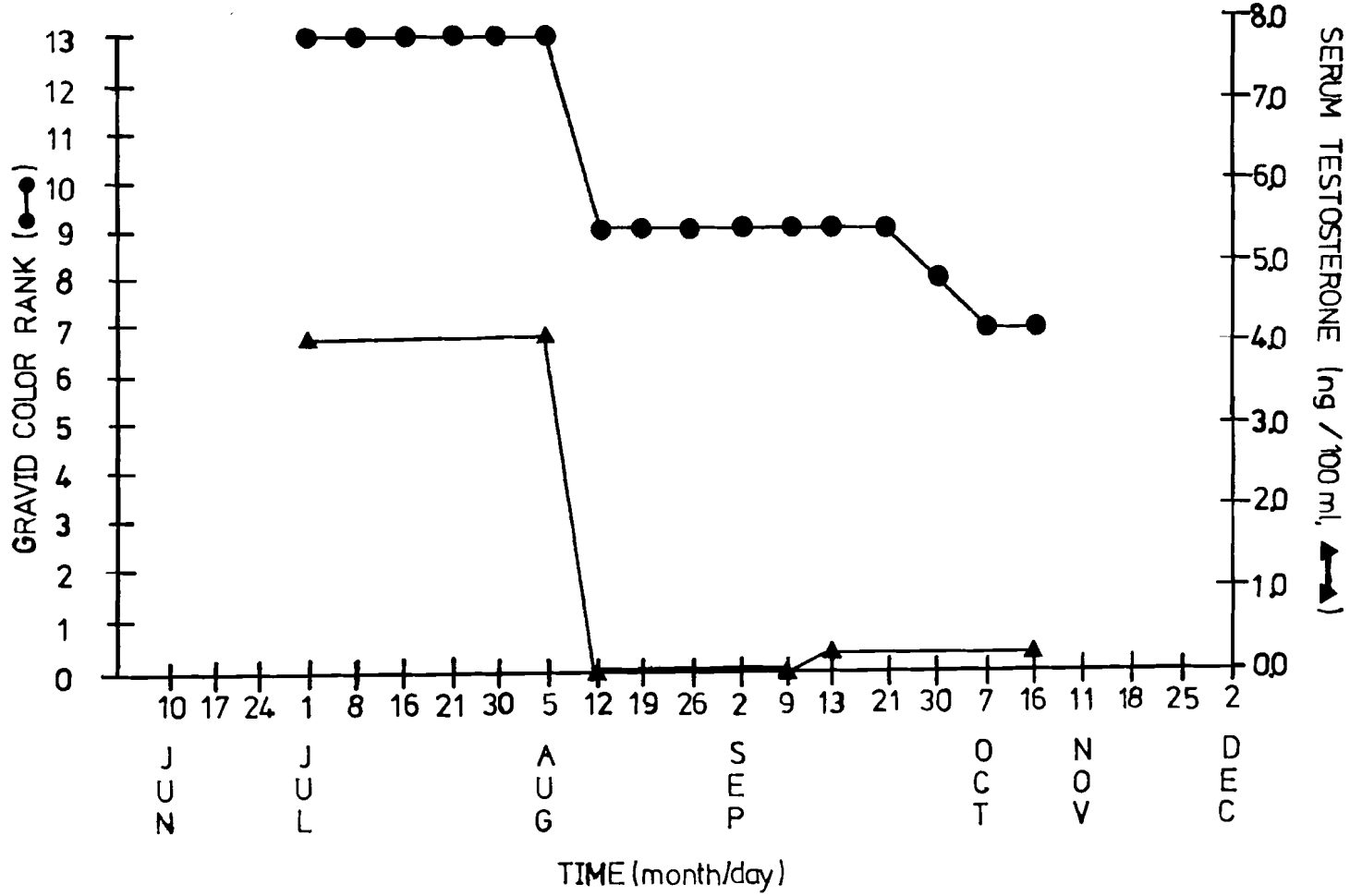
SEP
OCT
NOV
DEC
TIME (month/day)

Figure 5. The relationship between gravid coloration, serum testosterone, and time for C. collaris # 24.


GRAVID COLOR RANK (●)
SERUM TESTOSTERONE (ng / 100 ml, ▲)
TIME (month/day)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
10 17 24 1 8 16 21 30 5 12 19 26 2 9 13 21 30 7 16 11 18 25 2
JUN
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DEC

Figure 6. The relationship between gravid coloration, serum testosterone, and time for C. collaris # 33.

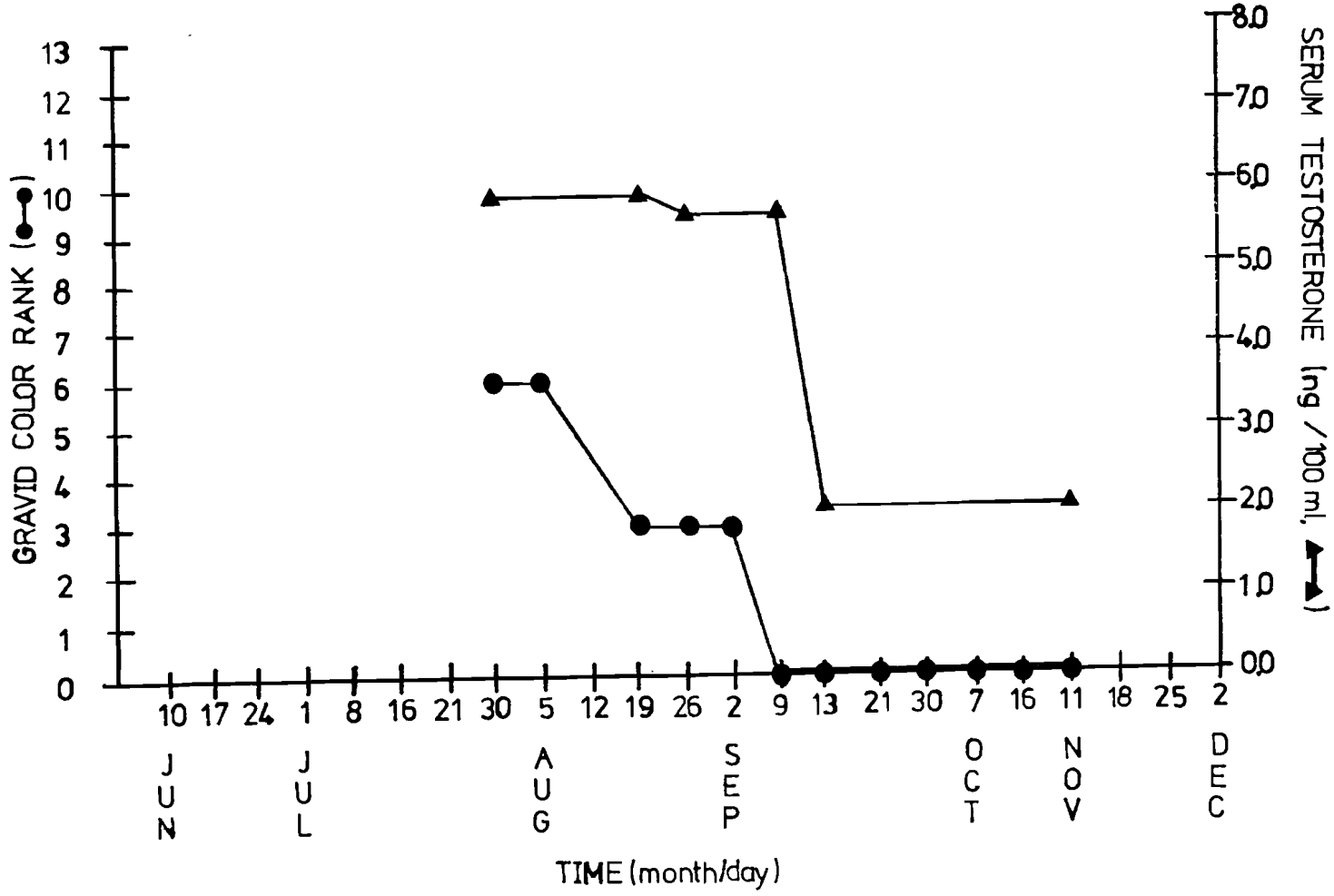
GRAVID COLOR RANK (●—●)
SERUM TESTOSTERONE (ng / 100 ml, ▲—▲)
TIME (month/day)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
10 17 24 1 8 16 21 30 5 12 19 26 2 9 13 21 30 7 16 11 18 25 2
JUN
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Figure 7. The relationship between gravid coloration, serum progesterone, and time for C. collaris # 12 (control).

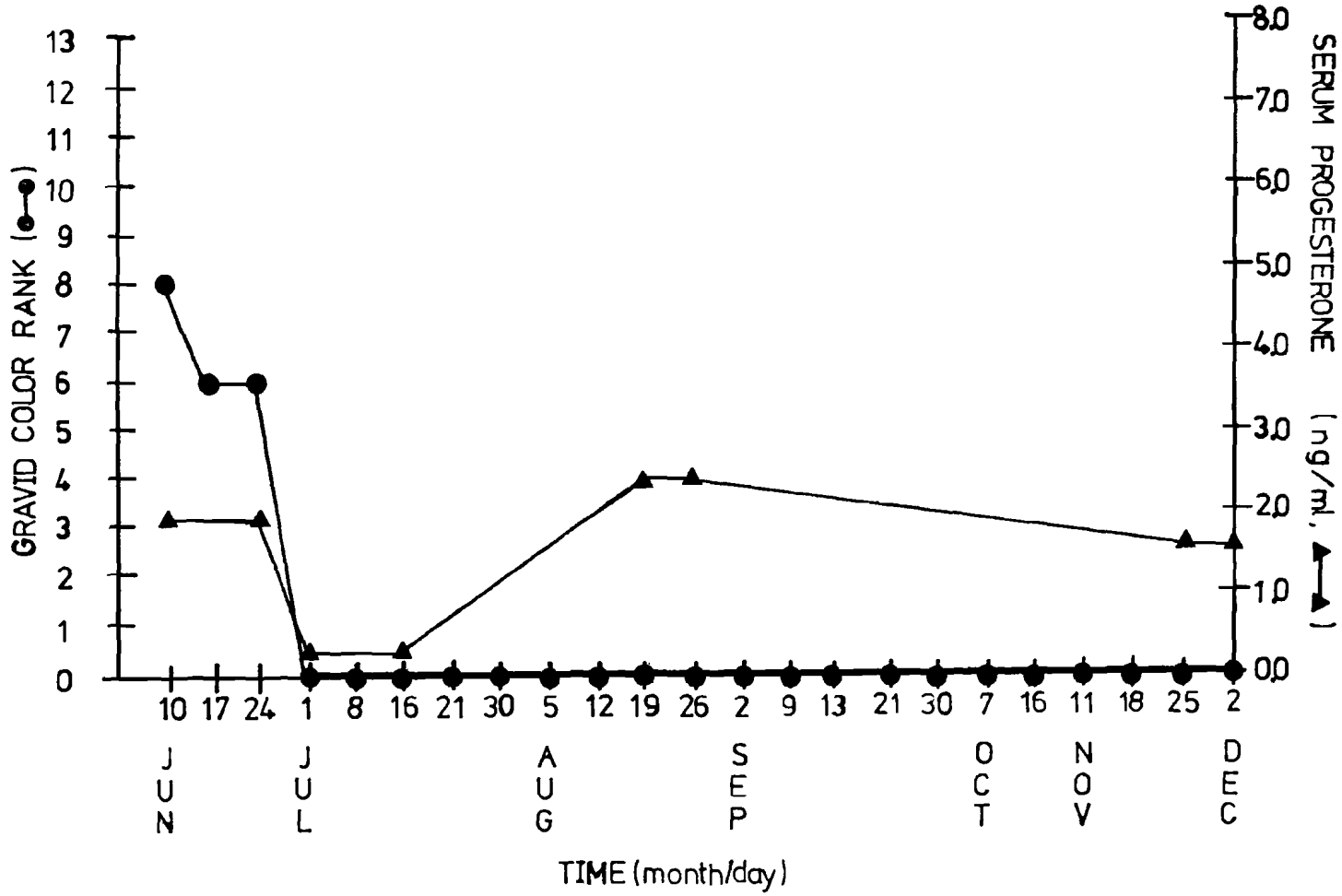
GRAVID COLOR RANK (●)
SERUM PROGESTERONE (ng/ml, ▲)
TIME (month/day)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
10 17 24 1 8 16 21 30 5 12 19 26 2 9 13 21 30 7 16 11 18 25 2
JUN
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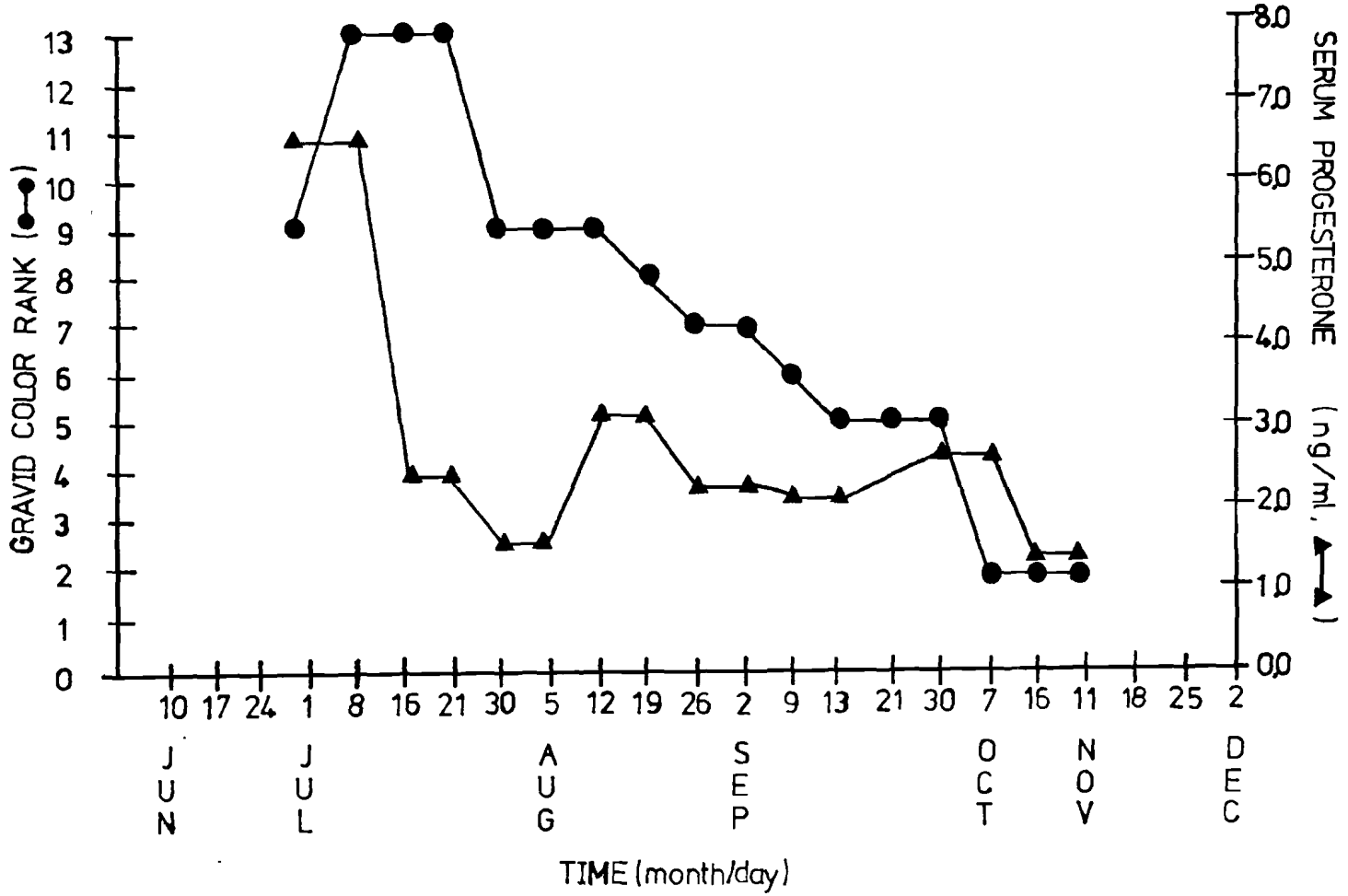
Figure 8. The relationship between gravid coloration, serum progesterone, and time for C. collaris # 23.


GRAVID COLOR RANK (●—●)
SERUM PROGESTERONE (ng/ml, ▲—▲)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
0.0
1.0
2.0
3.0
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5.0
6.0
7.0
8.0
10 17 24 1 8 16 21 30 5 12 19 26 2 9 13 21 30 7 16 11 18 25 2
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TIME (month/day)

Figure 9. The relationship between gravid coloration, serum progesterone, and time for C. collaris # 26.

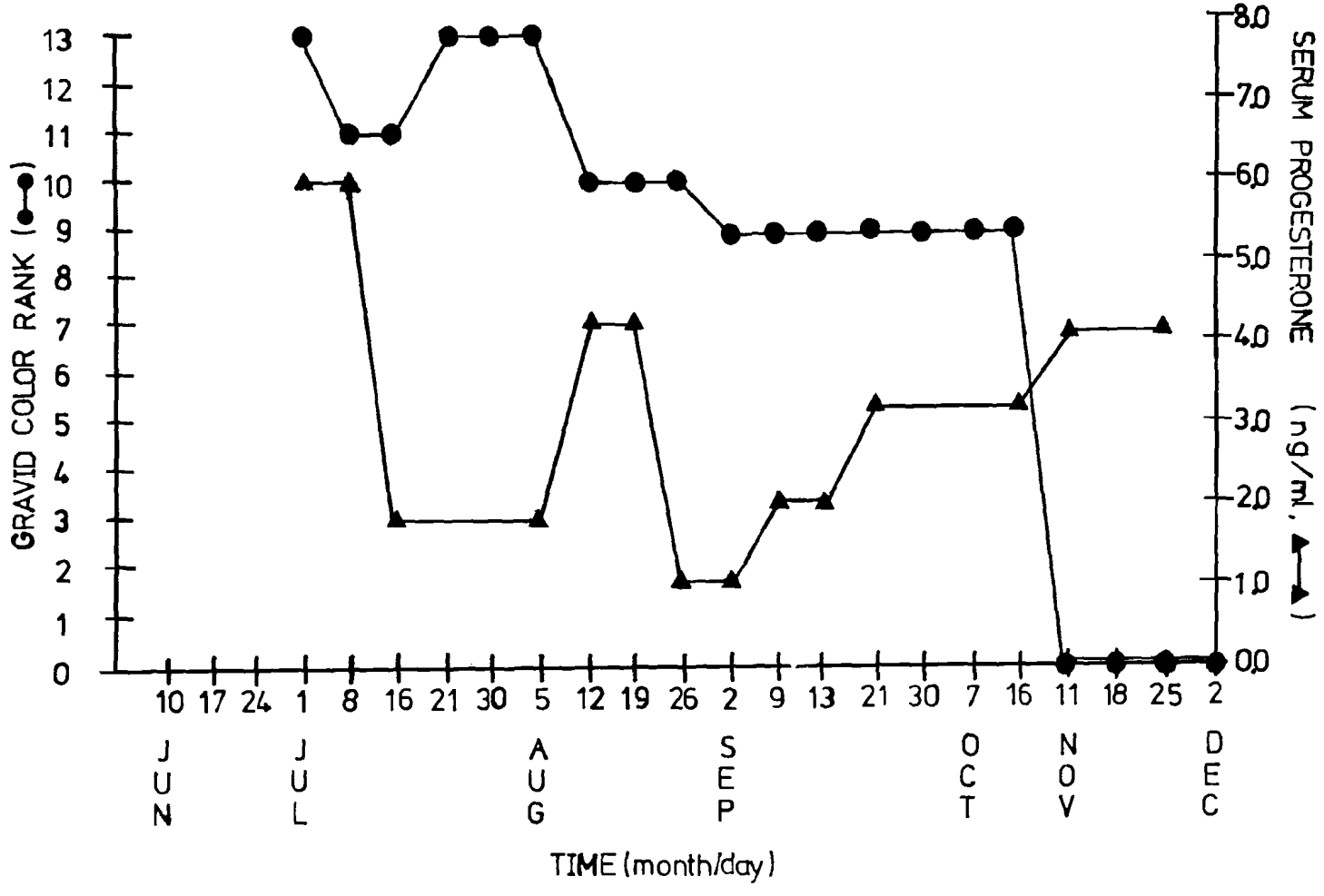
GRAVID COLOR RANK (●—●)
SERUM PROGESTERONE (ng/ml, ▲—▲)
TIME (month/day)
13
12
11
10
9
8
7
6
5
4
3
2
1
0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
10 17 24 1 8 16 21 30 5 12 19 26 2 9 13 21 30 7 16 11 18 25 2
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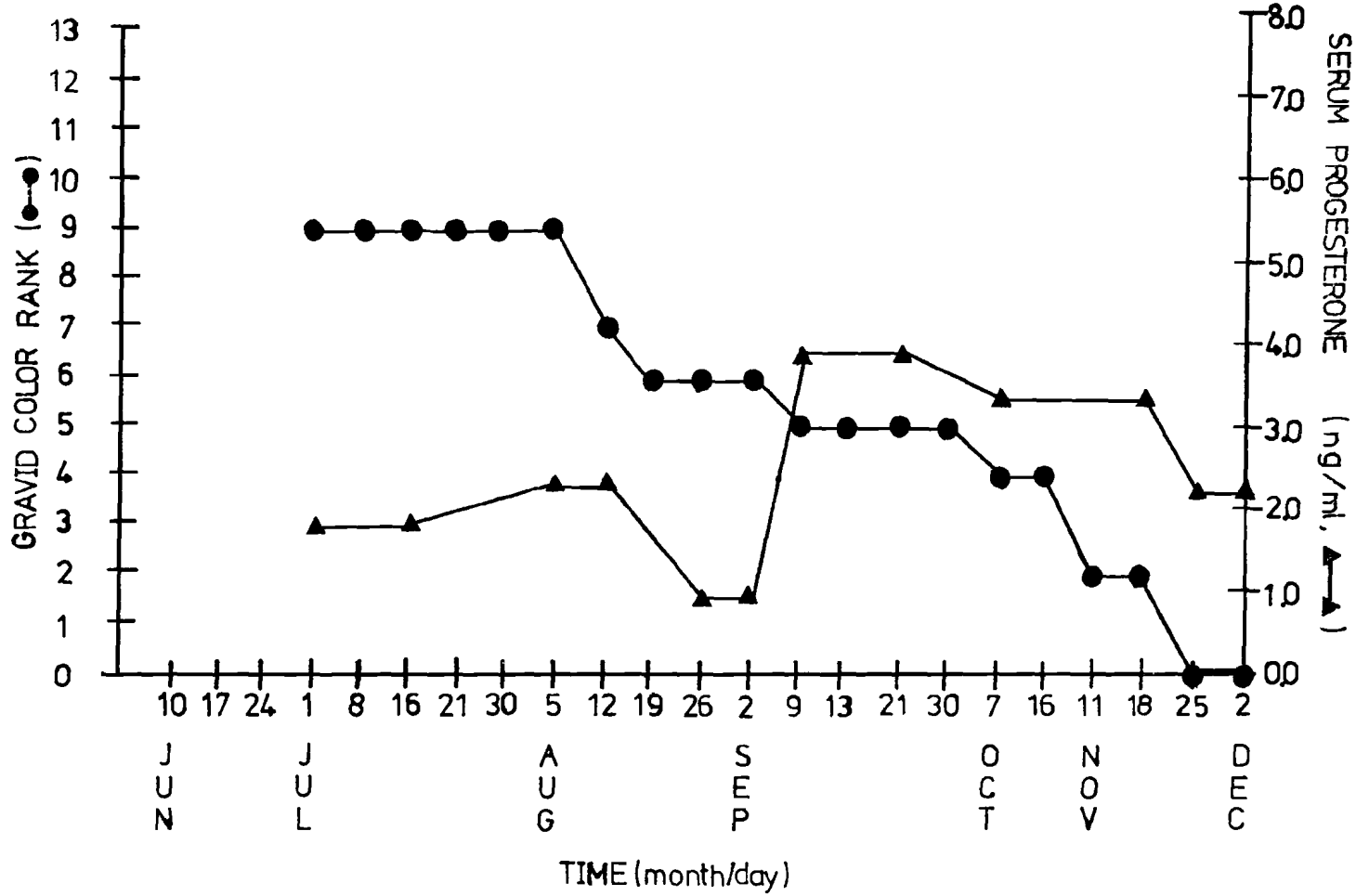
Figure 10. The relationship between gravid coloration, serum progesterone, and time for C. collaris # 28.


GRAVID COLOR RANK (●)
SERUM PROGESTERONE (ng/ml, ▲)
TIME (month/day)
0
1
2
3
4
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6
7
8
9
10
11
12
13
0.0
1.0
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8.0
10 17 24 1 8 16 21 30 5 12 19 26 2 9 13 21 30 7 16 11 18 25 2
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Figure 11. The relationship between gravid coloration, serum progesterone, and time for C. collaris # 31.

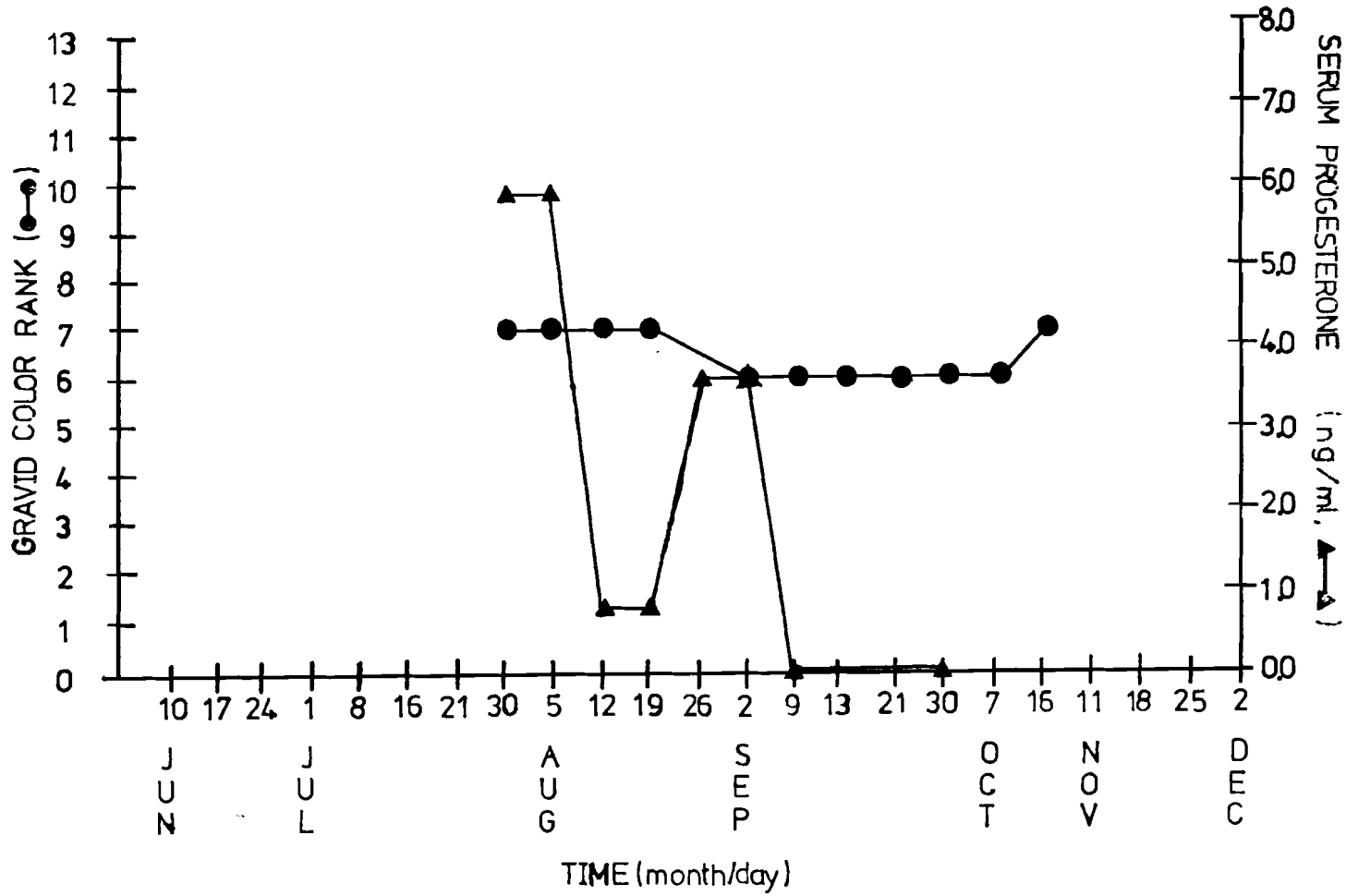
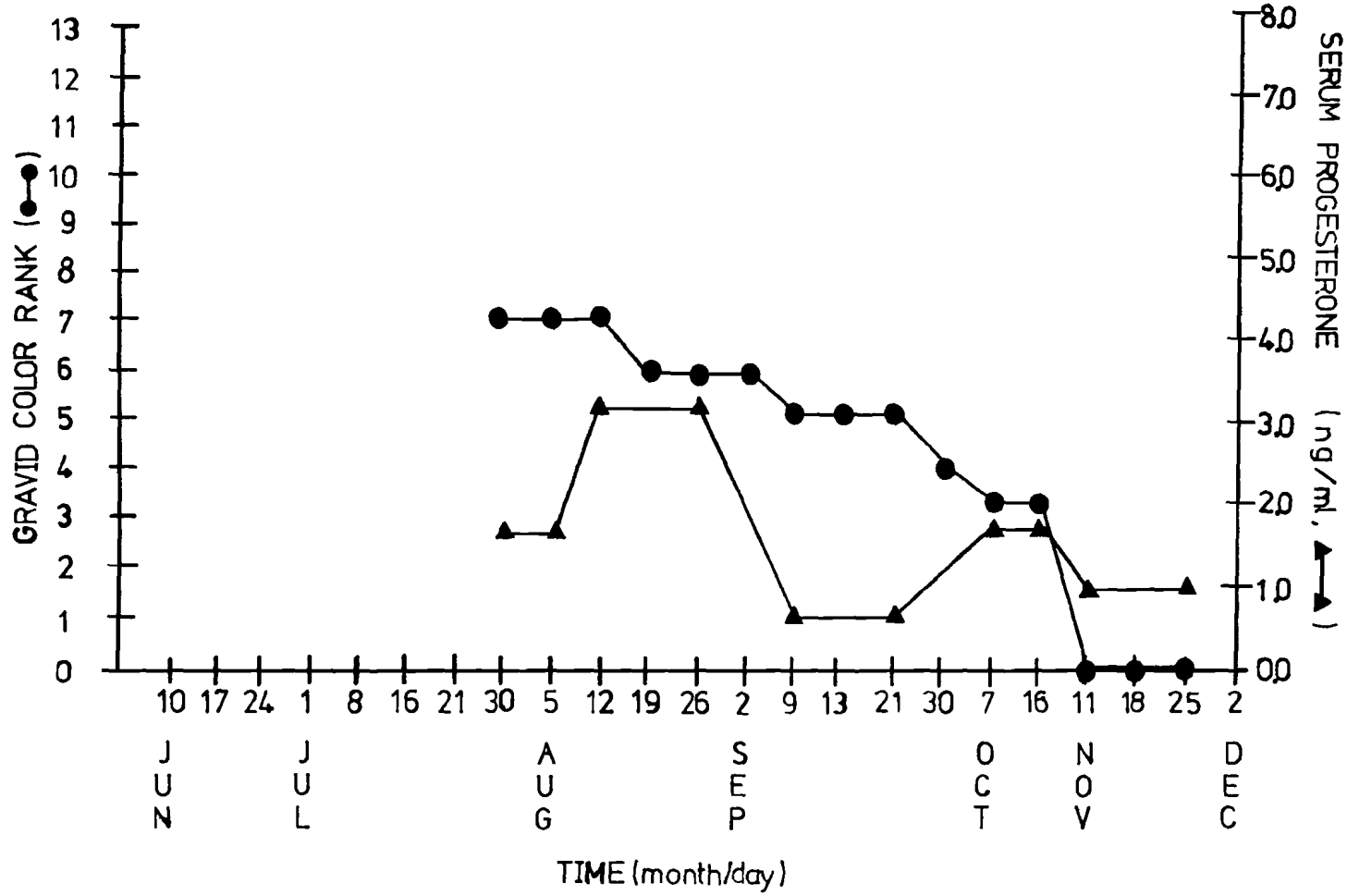

GRAVID COLOR RANK (●)
SERUM PROGESTERONE (ng/ml, ▲)
TIME (month/day)
0 1 2 3 4 5 6 7 8 9 10 11 12 13
0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0
10 17 24 1 8 16 21 30 5 12 19 26 2 9 13 21 30 7 16 11 18 25 2
JUN JUL AUG SEP OCT NOV DEC

Figure 12. The relationship between gravid coloration, serum progesterone, and time for C. collaris # 32.


GRAVID COLOR RANK (●)
SERUM PROGESTERONE (ng/ml, ▲)
TIME (month/day)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
0.0
1.0
2.0
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4.0
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8.0
10 17 24 1 8 16 21 30 5 12 19 26 2 9 13 21 30 7 16 11 18 25 2
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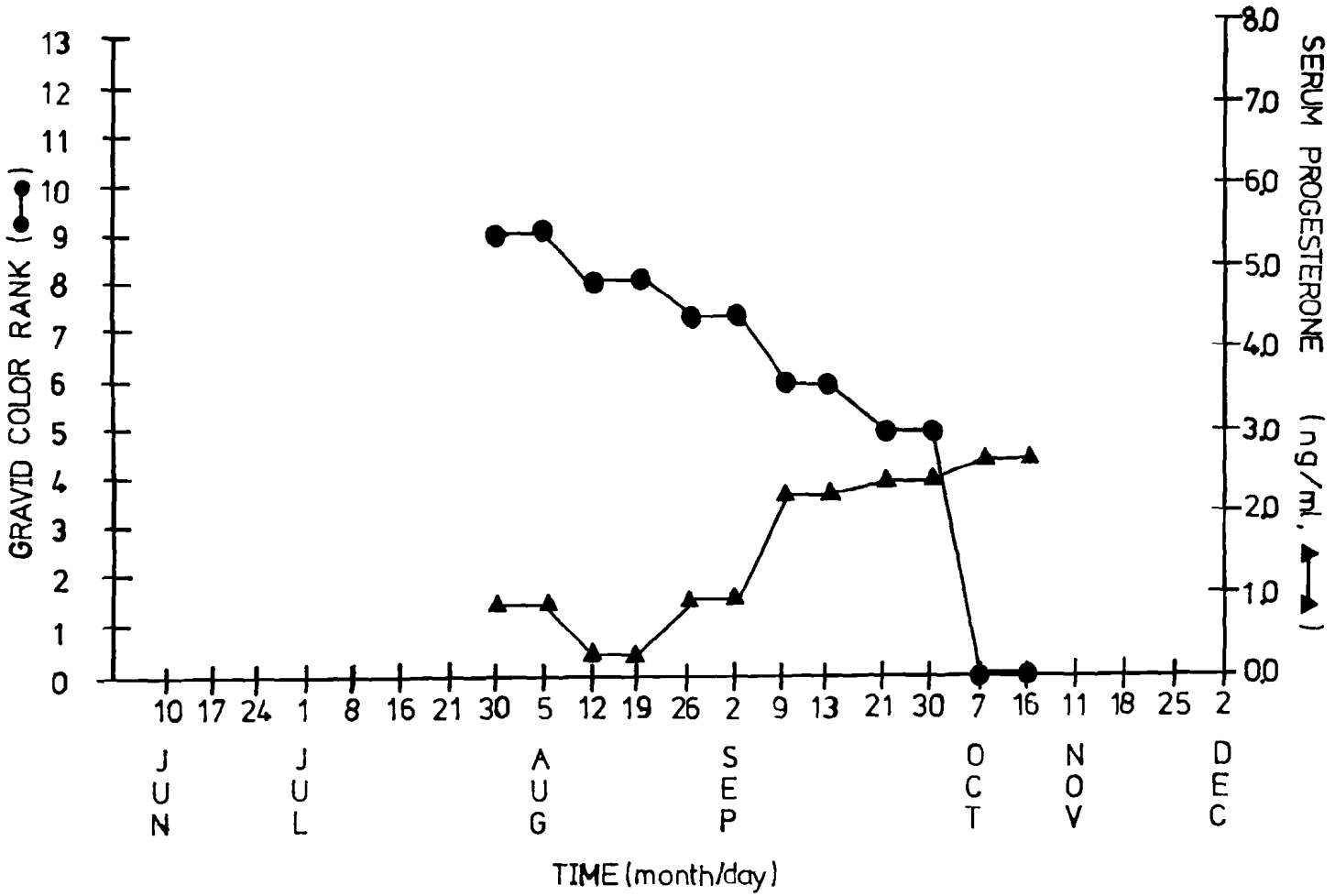
Figure 13. The relationship between gravid coloration, serum progesterone, and time for C. collaris # 34.


GRAVID COLOR RANK (●)
SERUM PROGESTERONE (ng/ml, ▲)
0
1
2
3
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5
6
7
8
9
10
11
12
13
0.0
1.0
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6.0
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8.0
10 17 24 1 8 16 21 30 5 12 19 26 2 9 13 21 30 7 16 11 18 25 2
JUN
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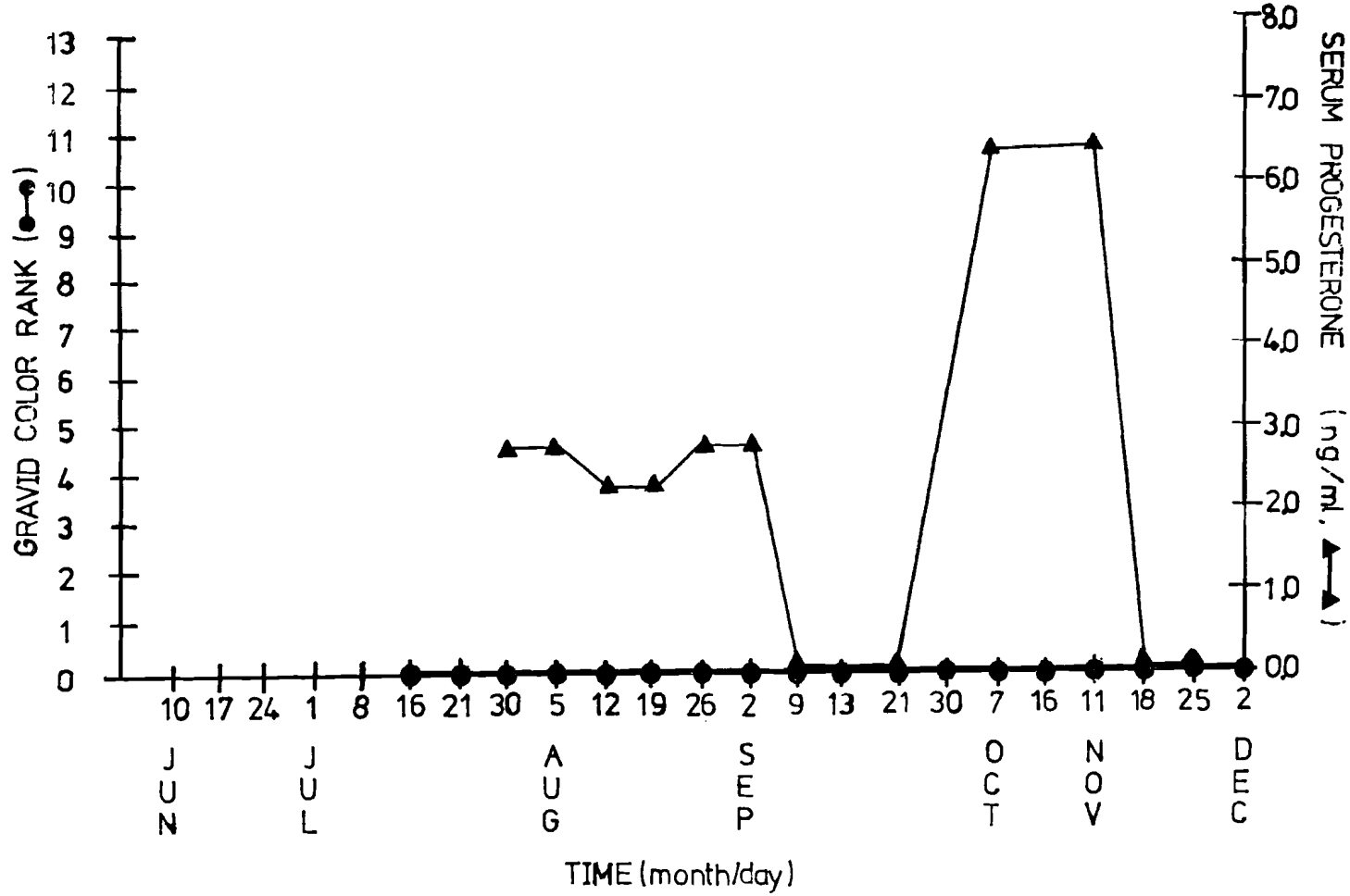
TIME (month/day)

Figure 14. The relationship between gravid coloration, serum progesterone, and time for C. collaris # 60 (control).


GRAVID COLOR RANK (●)
SERUM PROGESTERONE (ng/ml, ▲)
TIME (month/day)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
10 17 24 1 8 16 21 30 5 12 19 26 2 9 13 21 30 7 16 11 18 25 2
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stress due to captivity. Observations of captive Crotaphytus collaris females have been made in which they retained their gravid markings weeks after oviposition. One female was also observed to come out of hibernation with orange-red coloration (Yedlin and Ferguson 1973). No similar cases are reported in non-captive lizards.

Under field conditions orange-red coloration appears three to four days after copulation (Fitch 1956). About eight days after appearing, the gravid spots reach maximum brightness (Mosley 1963). Oviposition follows about one week later, with colors fading and barely visible two weeks after egg laying (Mosley 1963). The gravid colors last a total of about 30 days from appearance to fading in Crotaphytus collaris.

Cardiac Puncture

Although the cardiac puncture technique had been used on live lizard specimens (Arslan et al. 1978), no information could be found on its use for blood collecting from specimens maintained over any period of time. Crotaphytus collaris specimens under 15.0 g did not respond well to cardiac puncture. Two specimens died within 24 hours and two others exhibited lethargic symptoms for two to three days following the punctures. They died within the week. This resulted in the removal of all lizards 15.0 g and under from the study.

All lizards from which blood was drawn regularly developed tough, thickened epidermal areas around the puncture area. These areas appeared to be scar tissue. Once present, the scar tissue sizes did not change throughout the course of the investigation. These areas did not appear to be tender to the touch or have any adverse effect on the

lizards. They did not hinder cardiac punctures. It is not known if the scar tissue changed the components of the blood collected.

Protection Behavior

It is not know if Crotaphytus collaris lizards are monogamous or if they ever mate for life (Yedlin and Ferguson 1973). Fitch (1956) observed a male and female sunning together. They were also observed to move away from danger together.

On June 15, 1984, a gravid female was observed sunning with a male. When approached, the male displayed and then ran about 10 feet away from the female. He stopped two or three times. The female never moved. An attempt was made to determine if the male's actions were only for self-protection or if he was trying to protect the female. Movement by the observers was made back towards the female. The male immediately ran back to the female, placed himself between the female and the observers, and displayed. When the observers again moved toward the male, the male ran away from the female, presumably to lure the danger away. The observers left the area and returned after about thirty minutes and again noted the female and the male sunning together (personal observation).

That these two lizards were in the same territory lead to the conclusion that they were mates. No other females were observed in this area. Females do not leave their home ranges to mate (Stamps 1983) so male territories usually completely enclose the female's. To allow mating with two females, the male's range would have to at least partially overlap both females' territories (Stamps 1983). Not enough

evidence was available to prove or disprove that the observation presented was a monogamous situation.

There is also not enough evidence to explain why the male appeared to "protect" the female. He may have been insuring survival of his own genes. More observations will have to be done to reach any conclusions about the function of this protection behavior and to find if it is a standard behavior of Crotaphytus collaris.

Oviposition

Crotaphytus collaris are thought to deposit one clutch of eggs per season in Kansas (Fitch 1956; Ferguson 1976), as opposed to production of two clutches in Texas (Ballinger and Hipp 1985). Clutches average about seven to eight eggs (Ballinger and Hipp 1985). Females lay their eggs about seven days after maximum brightness of color spots has been attained (Mosley 1963). Although Mosley (1963) stated that females lay eggs from mid-June through early August (Smith 1946), Ferguson (1976) found that maximum orange color was present in June, although most females oviposit between July 16 and July 23.

Only 19 eggs were found deposited by the 13 lizards exhibiting orange-red coloration in this study. Some of the females may have oviposited before capture. This is possible because color comparison with Ferguson's (1976) information indicated that seven of the lizards with orange colors were in postgravid stages. The eggs were found between July 21 and August 1, 1984. Of the eggs found only ten were actually deposited under rocks or in protected holes. All the rest were deposited above ground in unprotected areas. The unusual

oviposition behavior was probably due to confinement conditions.

One female was observed to behave aggressively toward any threat near her deposited eggs. This is consistent with the findings of Yedlin and Ferguson (1973) that postoviposition aggression is used to defend nest sites. From the eggs located, four juvenile lizards were found. One was presumed eaten before removal from the cage, one escaped between the cage walls, and one died within eight days of unknown causes. The fourth one survived for approximately one month before he died. Other studies could be done on the mortality rate of confined juvenile Crotaphytus collaris.

Serum Testosterone Levels

Only two of the six lizards used for the RIA testosterone study had levels high enough to be detected (see Fig. 5 and Fig. 6). In the first two lizard specimens, the mean testosterone concentration was 0.033 ng/ml. This was lower than the finding by Arslan et al. (1978) of 1.570 $\pm$ 0.350 ng/ml plasma concentrations in gravid Uromastix hardwicki. Judd et al. (1976) found a mean of 0.258 $\pm$ 46 ng/ml in nongravid female Iguana iguana. Cooper and Crews (1988) found a total androgen concentration in bright females of 9.32 $\pm$ 2.06 ng/ml. It was postulated that the white layer did hold some testosterone and made it unavailable for analysis. The testosterone study could not be repeated because of lizard blood serum unavailability. More investigation must be done before conclusions can be made, since four of the testosterone hormone studies were under the sensitivity of the assay.

Some changes could be made if the procedures are repeated. The

shaken methylene chloride mixture should be filtered and the filtrate collected instead of centrifuging and aspirating the mixture. This would eliminate protein carryover. The insoluble lipid protein layer probably held any testosterone that did not get extracted, since testosterone is very soluble in lipids. The use of petroleum ether to extract the hormone instead of methylene chloride may help. It is unknown how the cardiac puncture method for drawing blood compares to drawing blood from the tail vessels (Judd et al. 1975). No information is available about the use of either method on lizards kept over long periods of time.

Data from specimens # 8, # 17, # 22, and # 30 indicate that there is not a correlation between the gravid colors and the testosterone levels. These specimens exhibited some orange-red coloration at times when hormone levels were undetectable. However, there is a pattern between the gravid colors and the testosterone levels in specimen # 24 and # 33. Gravid colors decreased in brightness as the hormone levels dropped (see Fig. 5 and Fig. 6). The data from these two lizards support the findings that testosterone injections induce gravid coloration in Crotaphytus collaris (Cooper and Ferguson 1972) and in Holbrookia propinqua (Cooper and Clarke 1982). It is hypothesized that the orange colors will also become more intense as hormone levels increase. Testosterone injections produced a significant increase in orange intensity when compared to control specimens of Crotaphytus collaris (Cooper and Ferguson 1972). More studies are necessary.

Elevated testosterone levels increased aggressive behavior in gravid lizards (Cooper and Clarke 1982). Fitch (1956) noted that

gravid Crotaphytus collaris became aggressive and most rejected copulation attempts when orange spots appeared. They were most aggressive when their colors were brightest. Cooper and Crews (1988) found a different situation in keeled earless lizards. Females that were in the beginning stages of brightening accepted copulation attempts and were less aggressive than completely bright females. Fitch (1956) is supported by Ferguson (1976) who shows that coloration is brightest when corpora lutea are present in the ovaries and by Arslan et al (1978) who showed that steroid concentrations of testosterone are highest in the luteal tissue compared to concentrations in the follicular tissue of both preovulatory and gravid Uromastix hardwicki.

The correlation between gravid coloration, aggressiveness, and testosterone in female Crotaphytus collaris follows what is know about testosterone in male lizards. Cooper et al. (1987) found that testosterone injections induced orange sexual coloration and aggressive behavior in male Eumeces laticeps. They also found that testes are enlarged during breeding season when copulation and aggressive behaviors are highest. When testicular size is reduced, these social behaviors also decrease (Cooper et al. 1987). It is conceivable that testosterone could cause gravid coloration and aggressive behavior in female lizards, also.

Serum Progesterone Levels

In the present study, progesterone levels were higher than testosterone levels in gravid lizards. This was also noted by Arslan

et al. (1978). The mean progesterone level Crotaphytus collaris with gravid coloration was 2.07 ng/ml ± 0.75. This is low compared to the results of Arslan et al. (1978) on gravid Uromastix hardwicki of 13.410 ng/ml ± 1.430. It is also lower than the 34.70 ± 6.61 ng/ml concentration in keeled earless lizards (Cooper and Crews 1988).

The low progesterone levels may be due to incomplete extraction. Only five milliliters of petroleum ether were added during the extraction phase instead of 20 ml. Some of the progesterone was probably left in the sample after extraction. The difference among the three studies may also be due to the date when the blood was drawn. Arslan et al. (1978) used serum from blood collected in May, the early part of the reproductive cycle for Uromastix hardwicki. In the spiny-tailed lizard, maturation of eggs and ovulation occurs in late April or early May (Arslan et al. 1976). Cooper and Crews (1988) collected their samples in April and May. The blood drawn in the present study was collected after June 9. Some of the gravid colors were already starting to fade. Ferguson (1976) shows in his study that June is the time when orange color is at its brightest. Corpora lutea are present in June, indicating that progesterone levels should be high during this time (Ferguson 1976, Arslan et al. 1978).

Findings that injected progesterone and testosterone do cause gravid coloration in Crotaphytus collaris (Cooper and Ferguson 1972) seem to indicate that intensity of color should increase as hormone levels increase. Levels of progesterone at the different color ranks were not consistent among lizards in the present study (see Fig. 7 through Fig. 14). It is possible that progesterone is a cause of

gravid coloration. Cooper and Ferguson (1972) found a significant difference, after 10 days, between specimens injected with progesterone and control Crotaphytus collaris. However, they did not find a significant difference between specimens injected with progesterone and those injected with testosterone.

Changes in the procedure may help increase uniformity of hormone measurements between lizards. The extraction method has already been mentioned. Perhaps known amounts of hormones could be injected into the lizards to increase levels, making it easier to find actual hormone levels in the serum or plasma. Lizard serum may need to be frozen at colder temperatures than 0 C. Arslan et al. (1978) stored Uromastix hardwicki blood at -15. (It is assumed that this is Celsius although the literature does not specify.) Cooper and Crews (1988) stored their samples at -20 C.

Mechanisms of Color Change

It has been established that gravid coloration in lizards is due to a physiological mechanism involving steroid hormones (Cooper and Ferguson 1972; Cooper and Clarke 1982). Physiological color changes involve dermal melanophores in which melanin is dispersed or aggregated in a time span of minutes to days (Gorbman et al. 1983). The effect of steroid hormones on melanophores is not fully understood.

Dermal chromatophore units are the structures involved in rapid color change. These units are comprised of xanthophores, lying below the epidermis, iridophores, which are underneath the xanthophores, and the bottom melanophores. The melanophores contain processes that

extend up through the layers and end below the epidermis (Smith 1946; Taylor and Hadley 1970). Xanthophores contain red, yellow, and orange pigment and iridophores contain reflective guanine crystals. Melanophores contain phaeolomelanins, the yellow-orange melanins, and eulamins, the black and brown melanins. The melanophores are the elements which undergo change in the color system, according to Smith (1946). All other structures are stationary filters or reflectors. Xanthophores and iridophores are exposed to view or covered by action of the melanophores (Parker 1948). Gorman et al. (1983) and Turner and Bagnara (1971) state that frog iridophores move in response to hormones, although Taylor and Hadley (1970) show that this does not occur in Anolis carolinensis.

MSH is the hormone controlling the dermal chromatophore units. Decreased MSH levels cause dispersion of guanine crystals and aggregation of melanin under the iridophore layer. Light then reflects off of the iridophores and through the xanthophores, resulting in a lighter colored animal. Increased MSH results in the animal becoming darker (Gorbman et al. 1983). In known systems, a melanophore-inhibiting factor (MIF) is controlled by the amount of light information received by the hypothalamus. Increased light causes MIF to be released which, in turn, inhibits MSH release in the pars intermedia. Acetylcholine also causes melanin dispersion; and epinephrine causes melanin to aggregate (Smith 1946; Gorbman et al. 1983) Epinephrine will lighten or darken an animal, depending on the types of receptors located on the chromatophores. Stimulating alpha-adrenergic receptors causes color to lighten and stimulating beta-

adrenergic receptors causes color to darken (Gorbman et al. 1983).

It is possible that iguanid gravid color change is based on dermal chromatophore units. There is no information on how steroid hormones affect pigment cells and whether this effect is direct or indirect. The following hypotheses could be explored:

1) increased progesterone and/or testosterone levels stimulate MIF production to decrease MSH action,
2) increased progesterone and/or testosterone levels stimulate epinephrine action on melanophores,
3) increased progesterone and/or testosterone levels act directly on the melanophores to increase phaelomelanin production,
4) the role of captivity and handling on 21 C steroid levels,
5) if alpha-adrenergic receptors are located on pigment cells found in body areas with orange-red color.

There is evidence that follicle-stimulating hormone (FSH) can induce gravid color changes by causing production of estrogen and progesterone (Medica et al. 1973). Licht (1970) found that luteinizing hormone (LH) can also cause orange-red lateral spots, although he thought there was some FSH contamination. There is disagreement whether lizards have one gonadotropin or two as mammals do. If lizards do only have an FSH-like substance, it would have to be controlled by estrogen feedback so ovulation can be prevented but not ovary growth (Callard et al. 1972). Chan and Callard (1974) caution that hypophysectomized animals do not fully respond to LH injections and

data from investigations should not be used to prove the lack of LH in reptiles.

It has been shown that estrogen injections can enhance gravid coloration produced by progesterone (Cooper and Ferguson 1973). This has also been effective when estrogen and progesterone have been injected together (Medica et al. 1973). There is no information about estrogen's priming effect with testosterone. Even though progesterone and testosterone are both found in luteal material (Arslan et al. 1978), it is doubtful that estrogen would increase color change due to testosterone, since estrogen and testosterone frequently antagonize each others actions. It is unknown whether progesterone and testosterone work together to produce gravid color change. This does seem possible since their serum concentrations rise during the gravid state. It may be that one acts on the melanophores and one on the iridophores to produce the gravid colors.

Gravid Color Function

There is controversy over the function of lizard gravid coloration. Researchers seem to agree that orange-red lateral spots help indicate the female sex (Vinegar 1972; Cooper 1984). Greenberg (1945) found that the yellow-orange gular region of the male was a significant recognition feature. He also found that males not displaying the yellow-orange color would be courted. The presence of orange coloration in particular body areas may advertise a particular sex, as well as a lizard not receptive to male courtship advances.

It has been suggested that gravid coloration inhibits male

copulation attempts (Cooper and Ferguson 1972). In Holbrookia propinqua the yellow coloration induces courtship behavior more frequently than the orange color (Cooper 1986). Orange coloration does not inhibit male attempts at copulation in Crotaphytus collaris (Fitch 1956; Medica 1973; Cooper 1986, personal observation). This could be due to high testosterone levels in males, which cause attempted copulation with females regardless of receptivity (Cooper et al. 1987). Brightening keeled earless lizards were likely to be sexually receptive, unlike females that did not have gravid colors or those which were completely bright (Cooper and Crews 1988). This does not appear to be the case in collared lizards.

Gravid colors are accompanied by behavioral postures such as sidlehopping (Fitch 1956; Vinegar 1972). Aggressive displays during gravidity are different than threat displays used at other times. These behaviors may indicate to males that the female is gravid and probably will reject courtship attempts, but that the females should not be considered a threat (Clarke 1965; Yedlin and Ferguson 1973; Vinegar 1972). Males rejected by gravid females appear tolerant to the aggressive behavior (Clarke 1965; Vinegar 1972; Cooper 1986). There is no information about how males react to nongravid female rejections.

Different opinions also exist about increased exposure to danger that gravid coloration represents. Cooper (1984) and Shine (1980) feel that orange-red coloration makes the females more visible to predators. Cooper (1986) speculates that some benefit of the colors must outweigh the increased risk of predation. Clarke (1965) indicates that the orange coloration is hidden from predators and shown only to males

attempting copulation in Holbrookia propinqua. Mosley (1963) suggested that bright coloration may have an inhibitory effect on predators. Other amphibians and reptiles do use their bright colors to fool or warn predators (Porter 1972).

Further studies need to be done. These studies should include data regarding male behavior toward gravid and nongravid females that reject copulation attempts. The effects of gravid colored models on predators should be compared to models displaying regular color patterns. Males' responses to varying degrees of gravid color intensity should be recorded. The appearance of orange-red spots should also be investigated. It needs to be determined if the spots appear before or after copulation or if it varies among species. These kinds of investigations would help determine if gravid color functions vary for different lizard species. It would also settle differing viewpoints that the coloration is used to signal already gravid females (Fitch 1956) as opposed to signaling that the females were ready for fertilization (Medica et al. 1973). Although it is not probable that the orange-red spots have an inhibitory effect on predators, no information is available on mortality rates of females during the gravid state.

SUMMARY

Evidence suggests that the orange-red gravid coloration of lizards is caused by progesterone and testosterone. This investigation attempted to find if any relationship existed between the gravid color levels and the hormone levels in female Crotaphytus collaris. It was hypothesized that increases and decreases in color levels would follow the same changes in steroid levels. Similar patterns between the gravid colors and testosterone levels were found by this investigation. Data correlating progesterone levels and gravid colors were inconclusive.

Cardiac punctures were physically damaging to Crotaphytus collaris that weighed about 15.0 g. Regular punctures produced a tough epidermal area around the puncture site. This scar tissue may have resulted in unwanted proteins or lipids becoming part of the serum samples. This may have contributed to the low detection of steroids from the RIAs.

Stress of captivity may have been the cause of prolonged gravid coloration. Gravid spots lasted until early fall in most specimens. Oviposition in unprotected areas areas may be the result of overcrowded conditions.

Dermal chromatophore units are thought to be the structures involved in gravid color changes in lizards. It is unclear how progesterone and testosterone act on the units. More investigation is necessary. More information is also needed to determine the role gravid colors play in lizard behavior. Orange colors, accompanied by

aggressive displays, seem to indicate that the female is in a gravid state, will resist copulation attempts, but is not a threat to males.

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APPENDIX A

APPENDIX A

WEIN LABORATORY PROGESTERONE PROCEDURE

1. Extract unknowns, control sera, and reagent (water) blanks.

   a. Place 400 ul of unknown serum/heparinized plasma or control serum in labeled 30 or 50 ml centrifuge tubes. For normal men or normal women in the follicular phase of the menstrual cycle, set up extraction tubes in duplicate.
   b. Add 2.0 ml of distilled water to each tube.
   c. Set up reagent (water) blanks in duplicate by placing 2.0 ml of distilled water in each of two 30 or 50 ml centrifuge tubes.
   d. Add 20 ml of Petroleum Ether to all tubes.
   e. Shake all tubes vigorously for 60 seconds.
   f. Centrifuge all tubes for 5 minutes at high speed (2000 X G).
   g. Label glass test tubes, 16 X 125 mm, for each unknown serum, control serum, and reagent blank.
   h. Transfer an appropriate aliquot of the upper layer from each centrifuge tube to its respective test tube:

      1) 10-15 ml — normal men; normal women (follicular phase); serum equivalent = 200-300 ul

      OR

      2) 2.5-5 ml — normal women (luteal phase); serum equivalent = 50-100 ul

      OR

      3) 100 ul - 1 ml — pregnant women (16-40 weeks); serum equivalent = 2-20 ul

      For additional references which may be helpful in determining the volume of extract to be used see the Expected Values section below.

   i. Evaporate all tubes to dryness in a warm water bath (40-50 C) with the aid of a stream of air or nitrogen. Standards may be dried simultaneously.

2. Prepare Standards

   a. Label glass test tubes, 16 X 125 mm, in duplicate: 0, 100, 250, 500 and 1000.
   b. Transfer 0, 10, 25, 50 and 100 ul of Progesterone Standard, at 20 C, to each tube, respectively, in duplicate.
   c. Evaporate to dryness as in step 1i above.
   d. The progesterone concentration is equivalent to 0, 100, 250, 500 and 1000 picograms per tube, respectively.

3. Radioimmunoassay

a. To all tubes, add 50 ul of ^{3}H-Progesterone. Mix well to dissolve.
   CAUTION; NEVER PIPET RADIOACTIVE MATERIAL BY MOUTH.
b. To all tubes add 1.0 ml of Phosphate Buffer, pH 7.4. Mix well.
c. To all tubes, while mixing on a vortex mixer, add 100 ul of Working Progesterone Antibody Solution.
d. Incubate all tubes in an ice bath (4-8 C) for 30 to 90 minutes. Sixty minutes is a good average time.
e. While in the ice bath, add 0.5 ml of cold Coated Charcoal Suspension, to all tubes. Mix well. Allow to stand for 5 minutes in the ice bath and then centrifuge at high speed (2000 X G) for 7 minutes.
f. Decant the clear supernatant solution into liquid scintillation vials, and add 10 ml of scintillation cocktail. Cap vials, mix well by shaking, and allow to stand for 30 minutes at room temperature in the dark before counting.
g. Count each tube for 2 minutes of 2 % efficiency in a Scintillation (Beta) counter with windows set for counting tritium (^{3}H).
h. Record counts per minute (CPM) for each tube.

APPENDIX B

APPENDIX B

WEIN LABORATORY TESTOSTERONE PROCEDURE

1. Extract unknowns, control sera, and the reagent (water) blanks in duplicate.

   a. Place 100 ul of male serum or 500 ul of female serum in polypropylene centrifuge tubes.
   b. For sample volumes up to 500 ul, add 0.5 ml distilled water to each tube. For sample volumes greater than 500 ul, add an equal volume of distilled water to each tube. Mix well.
   c. Set up reagent (water) blanks by placing 1.0 ml of distilled water in polypropylene centrifuge tubes.
   d. Add 10.0 ml of purified methylene chloride to all tubes.
   e. Shake vigorously for sixty seconds.
   f. Pour the entire contents into a funnel fitted with Whatman #1 PS filter paper, 9 cm diameter (phase separation paper) and collect the filtrate in a 16 X 100 mm glass test tube.
      OR Alternate Step f: Centrifuge for seven minutes at high speed (1500 X G). Aspirate off upper aqueous layer. (For those with limited experience in performing this procedure it is recommended the solvent be filtered with Whatman #1 filter paper to aid in the prevention of protein carry-over.)
      Transfer 4 ml aliquots into two 16 X 100 mm glass test tubes (equivalent to 40 ul serum for male and 200 ul serum for female).

2. Set up standard tubes in duplicate.

   a. Label glass test tubes: 0, 10, 25, 50, 75 and 100.
   b. Place 0, 10, 25, 50, 75 and 100 ul of Testosterone Standard in each test tube, respectively.
   c. The Testosterone concentration per tube is equivalent o 0, 100, 250, 500, 750 and 1000 pg, respectively.

3. Evaporate sample extracts and standards to dryness in a warm water bath (40-50 C) with the aid of a stream of air or nitrogen.

4. To all tubes, add 50 ul of ^{3}H-Testerone Solution, using a pipetting device. Mix well.
   CAUTION: NEVER PIPET RADIOACTIVE MATERIAL BY MOUTH.

5. To all tubes, add 1.0 ml of Phosphate Buffer, pH 7.4.

6. To all tubes, while mixing on a vortex mixer, add 100 ul of Testosterone Antibody Solution.

7. Incubate the tubes in an ice bath (4-8 C) for sixty minutes or longer.

8. Leaving the tubes in the ice bath, add 0.5 ml of cold Coated Charcoal Suspension to all tubes. Mix well. Allow to stand for five minutes in the ice bath and centrifuge at high speed (1500 X G) for seven minutes.

9. Decant the clear supernatant solution into liquid scintillation vials, and add 10 ml of scintillation cocktail. Cap and mix well by shaking and allow to stand for fifteen minutes at room temperature, in the dark, before counting.

10. Count for at least two minutes (or 2 % efficiency) in a Beta (scintillation) counter with windows set for counting tritium (^{3}H).

11. Record counts per minute (CPM) for each tube. (See NOTES on opposite page).

Kitty Sue Bobbitt
Signature of Graduate Student

Robert J. Clarke
Signature of Major Advisor

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11/29/88
Date

Steroid Levels and Gravid Coloration in Crotaphytus collaris

Title of Thesis/Research Report

Signature of Graduate Office Staff Member

Date Received