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Effects of temperature and photoperiod on hemolymph vitellogenin levels during spawning events of the blue crab, *Callinectes sapidus*, in captivity

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Abstract

Vitellogenesis is the process of production and uptake of vitellogenin (VtG), the precursor of vitellin (VT = yolk protein), for ovarian development. In *Callinectes sapidus*, hemolymph VtG levels are positively correlated with ovarian development. We aimed to develop an invasive tool to monitor ovarian development in order to predict spawning. The present study determined the effects of photoperiod and temperature conditions on levels of hemolymph VtG of the females in captivity kept in a closed recirculating system. Hemolymph VtG levels measured using a competitive ELISA correlates positively with temperature (21° versus 11°C) but are negatively correlated with photoperiod (0L:24D and 8L:16D versus 16L:8D and 24L:0D). Also, heavier (larger) females contain more VtG in their hemolymph and produce greater numbers of larvae. At 21°C, the VtG levels show high individual variations and continuously change over time, indicating that VtG production and uptake by ovary may be continuous and dynamic over multiple spawning events in *C. sapidus*. Microsatellite analysis of these females reveals a great diversity within and between the batches, implying that resident *C. sapidus* populations in the Chesapeake Bay are genetically dynamic. Such a great genetic diversity could be responsible for the high variation in VtG levels in these females.

KEYWORDS
blue crab, competitive ELISA, genetic analysis, spawning, vitellogenesis, vitellogenin

1 | INTRODUCTION

Life histories of female crustaceans represent a range of species-specific reproductive strategies that are variable in ovarian development and spawning as well as moulting. Some species experience their pubertal and final moult upon reaching adulthood. This is the case for *Callinectes sapidus* whereby soft-shell virgin females mate at one discreet time in their life immediately after the pubertal-terminal moult with hard-shell males. *Chionoecetes opilio* and *Chionoecetes bairdi* have two mating patterns with both soft- and hard-shell mating (Elner & Beninger, 1995). Many other crustaceans including *Homarus americanus* (Byard & Aiken, 1984; Nelson, 1986), *Macrobrachium nipponense* (Okumura, Han, Suzuki, Aida, & Hanyu, 1992) and *Macrobrachium rosenbergii* (Derelle, Grosclaude, Meusy, Junera, & Martin, 1986; Okumura & Aida, 2001) continue to moult and mate even after reaching sexual maturity. In these species, the moult/

reproduction stages alternate causing cyclic variations in vitellogenin (VtG) levels that increase after moulting and decrease prior to ecdysis and spawning (during the reproductive cycles).

Vitellogenesis is the critical step in ovarian development for crustaceans, which can be divided into two phases: primary and secondary (Charniaux-Cotton, 1985; Lee & Walker, 1995; Quackenbush, 1986). The primary stage includes accumulation of glycoproteins in the oocytes causing the initial small growth of the ovaries (Charniaux-Cotton, 1985). The secondary phase is characterized typically by elevated hemolymph VtG levels and ovarian growth as vitellin (VT) accumulation (Lee & Puppione, 1988; Zmora, Trant, Chan, & Chung, 2007).

For adult female *C. sapidus* inhabiting the Chesapeake Bay, ovarian development depends on season (Thongda, Chung, Tsutsui, Zmora, & Katenta, 2015) for which temperature and photoperiod are the most likely factors that could affect spawning activities (Bembe, Liang, & Chung, 2017). As multiple spawners, these females produce multiple clutches under optimal spawning conditions at an average interval of ~47 days (Bembe et al., 2017; Dickinson, Rittschof, & Latanich, 2006; Hines et al., 2003). It has already been shown that there is a positive correlation between the size of females and the number of larvae hatched (Bembe et al., 2017) and may be linked to the ability to produce relatively large amounts of VtG.

Callinectes sapidus is a sentinel species in the Chesapeake Bay where a precipitous declining in its population has been reported in the last two-decades (Miller et al., 2005; Zohar et al., 2008). The population of *C. sapidus* in the Chesapeake Bay is stratified by life-stage and sex dependent opposite migration, i.e. recruitment of juveniles from the mouth to the upper Chesapeake Bay and adult females in the opposite direction (Williams, 1984). Despite high (45%–55%) annual harvest levels (Bunnell & Miller, 2005; Lipcius, Seitz, Seebo, & Colon-Carrion, 2005; Miller et al., 2005), this constant recruitment and migration result in high genetic variation within the population of the Chesapeake Bay (Feng, Willams, & Place, 2017).

The objectives of the current study are to: (1) determine to what degree VtG levels are maintained during spawning and across multiple spawning events using a competitive ELISA; (2) test the effect of temperature and photoperiod on VtG levels; and (3) determine the potential genetic diversity of the experimental animals. Over 19 weeks during a 2-year period, four separate experimental systems were evaluated by monitoring hemolymph VtG levels in two temperature regimes (21 and 11°C) and four photoperiods: 0L:24D, 8L:16D, 16L:8D and 24L:0D.

2 | MATERIALS AND METHODS

2.1 | Animals

Two batches of primiparous adult female crabs were caught in crab pots by a local waterman in the Chesapeake Bay (Maryland, USA) on (Batch 1) November 17, 2006 and (Batch 2) November 24, 2007 at the end of fall migration period and acclimated as described (Bembe et al., 2017).

2.2 | Experimental conditions

The experimental conditions were described previously (Bembe et al., 2017). In brief, Animals were individually tagged using heavy electrical wire with numbers and were stocked at a density of 4–5 crabs per tank (91 × 112 × 58 cm deep) holding 0.3 cubic meter of water (Bembe et al., 2017). They were exposed to two different temperatures and four photoperiods: at 21°C under 0L:24D (n = 15); 8L:16D (n = 10); 16L:8D (n = 13); 24L:0D (n = 5) and 11°C under 0L:24D (n = 10); 8L: 16D (n = 5); 16:8D (n = 5); 24L:0D (n = 5). Specifically, the exteriors of the 0L:24D tanks were draped with black plastic sheeting (Film-Gard, 0.15 mm) to prevent light penetration, and a red light (General Electric, 25 W; 0 Lux) was used during feeding and observation. Lighting intensities (0–18 Lux) were measured in each tank using a Milwaukee lux meter (SM700). Temperature and salinity were daily recorded, while ammonia and nitrite were measured every 2 days, and pH was monitored weekly. The crabs were fed a piece of squid (10–15 g wet weight) and a pelleted diet (EWOS Brood, 12 mm) during the experimental period.

2.3 | Hemolymph sampling and monitoring spawning

Hemolymph was sampled at a 1:1 ratio with a modified marine anticoagulant (pH 4.0) (Söderhäll & Smith, 1983) twice a week for 19 weeks during which spawning was monitored. The hemolymph samples were kept at −20°C and assayed for the levels of total protein and VtG.

2.4 | Vitellin purification

Vitellin was purified using *C. sapidus* ovary at stage 2 or 3. It was homogenized in 10 volumes of homogenization buffer (20 mM Tris buffer, pH 7.5) and 10% protease inhibitor cocktail (Sigma) using a polytron (Ultra-Turrax T25; Janke and Kunkel IKA Labortechnik) and centrifuged for 10 min at 8,000 *g*, 4°C. The supernatants were precipitated with 60% ammonium sulphate on ice for 1 hr then centrifuged at 16,000 *g*, 4°C for 20 min. The pellet was washed in homogenization buffer by centrifugation as above. The final pellet that was re-suspended in running buffer (0.01% EDTA in PBS and 10% protease inhibitor cocktail) was eluted on a size exclusion column (BioGel P-300 beads) with running buffer. Fractions (3 ml/each) were analysed on a 4%–15% gradient SDS polyacrylamide gel.

The two bands with the molecular weight of ~75 and 100 kDa representing subunits of VT (Zmora et al., 2007) were excised and electroeluted in a non-denaturing running buffer (Tris-Glycine, pH 8.3; Centrielutor-Micro-Eluter, Millipore and Centricon 30) according to the manufacturer's instructions. Vitellin was concentrated by centrifugation on a Centriplus filter (3 kDa exclusion) at 3,000 *g*, 4°C and stored in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0).

2.5 | Affinity purification of anti-VT serum

Dry CNBr-activated Sepharose beads (Sigma) were used for affinity purification of ant-VT serum by following the manufacturer's recommendation. In brief, ~0.25 g CNBr was resuspended in ice cold 1 M HCl (pH 3.0), kept on ice for 30 min, and centrifuged at 500 *g*, 4°C for 5 min. The beads were washed four times with the coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0) by centrifugation as above. The purified VT (~300 μg) was first conjugated to the beads by incubating the mixture for 4 hr at room temperature with gentle agitation on a shaker (20–30 rpm, Roto-Shake Genie; Scientific Industries). After the allocated mixing time, 1 ml of 1 M Tris buffer (pH 8.0) was added to the solution, incubated at room temperature for 2 hr on the shaker, and centrifuged at 500 *g*, 4°C for 5 min. The beads were then washed three times in PBS by centrifugation as above.

Anti VT serum (2 ml) that was generated against VT (Zmora et al., 2007) was mixed into beads and incubated at 4°C for 1–3 days. The mixture was transferred to a column (1 × 5 cm) containing CNBr-VT. After three washes with 1 ml cold PBS, the antibody was eluted using cold 1 M HCl (pH 2.5). Ten fractions (500 μl/each) were collected directly into tubes containing 100 μl 1 M Tris (pH 8.0) for neutralization. The antibody fractions were stored in glycerol (2:1 ratio) at 4°C. The original 2 ml antiserum yielded ~500 μg of purified anti-VT.

2.6 | Levels of hemolymph VtG using a competitive ELISA

Competitive ELISA was carried out to measure the levels of hemolymph VtG using affinity purified antibody (Zmora et al., 2007) and 3,3′,5,5′ Tetramethylbenzidine (TMB, KPL) for colour development. Each 96-well plate included a standard ranging 100–2,000 ng/ml and a reference hemolymph sample containing a mixture of hemolymphs. The EC_{50} values of competitive ELISAs were 397.7 ± 14.7 ng/ml (n = 93), where n was the number of plates. Samples which were out of the standard range of either <100 ng/ml or >2,000 ng/ml were re-assayed accordingly. The hemolymph VtG levels were presented as mean ± 1 *SE* μg/ml hemolymph.

2.7 | Total hemolymph levels

Total protein quantification was performed on hemolymph samples to evaluate the differences in VtG concentrations seen between the two batches of animals. Using the highest and lowest VtG concentrations per female (two samples per female) at a 50-fold dilution, protein concentrations were determined using a BioRad *DC* protein assay kit. The data were presented as mean ± 1 *SE* μg/ml hemolymph.

2.8 | DNA extraction and Microsatellite analysis

DNA was extracted from the hemocytes of individual hemolymph samples from the experimental animals (Bembe et al., 2017) that were spun at 10,000 *g* for 1 min using the Qiagen DNeasy Blood and Tissue Kit. The extracted nucleic acids were quantified using a NanoDrop 1000 spectrophotometer (FisherSci) and diluted to 10 ng/μl concentration. For this analysis, we included another batch of animals (Batch 3) that were obtained in a different season, April but the same year of Batch 2, in order to examine the population dynamics of *C. sapidus* within a year.

The repeat-rich region located in the 3′ UTR of the moult-inhibiting hormone (MIH) was for this study as it successfully characterized the brood stock females and their broods through discriminating multiple paternity and correlation with a phenotype (Allman, Williams, & Place, 2017). The deposited cDNA sequence GenBank U19764 was used as a reference and template from each individual was amplified using primers MIHFAMM13F2 and MIHR2 (Invitrogen, Table 1) as follows: 2 μl DEPC-treated water, 5 μl Promega 2× master mix (Madison WI, USA), 1 μl of each primer at 5 μM each and 1 μl template DNA using the following amplification conditions: initial denaturation at 95°C for 2 min; denaturation at 35 cycles of 95°C for 15 s; annealing at 54°C for 30 s and extension at 72°C for 1 min. A 15 min extension step at 72°C was used to ensure homogeneity in the polymerase tails the products. These products were dye-labelled with the FAMM13F primer (Applied Biosystems, Carlsbad, CA, USA, Table 1) at the following conditions: 4 μl DEPC-treated water, 5 μl Promega 2× master mix and 1 μl of primer at 5 μM using the same PCR conditions as above with the modifications of 10 cycles and an annealing temperature at 53°C. A portion of the amplified product (6 μl) was added to 9 μl Hi-Di™ formamide and 0.5 μl of LIZ500 ladder (Applied Biosystems) and was denatured at 95°C for 3 min then snap cooled on ice. These samples were then run and analysed using GENEMAPPER software version 3.4 (3130xl genetic analyzer; Applied Biosystems). All results and peak callings were curated manually.

TABLE 1 Primer sequences for microsatellite analyses

Primer	Primer sequence (5′-3′)	Tm (°C)
MIHFAMM13F2	CACGACGTTGTAAAACGACgtgatacaaagatgttgatgacgtg	63
mihr2	aacttttgctcttcagccactg	60
FAMM13F	6FAM-cacgacgttgtaaacgac	55

The uppercase sequence on MIHFAMM13F2 represents the M13 binding tail that does not directly bind to the MIH gene and is incorporated into the first round of PCR products.

2.9 | Statistical analysis

The VtG levels were presented as mean ± 1 *SE*, where *n* was the number of animals. The EC_{50} of each competitive ELISA was calculated with SIGMAPLOT (Version 12). The spawned females were analysed using two-way ANOVA. Female weight, number of hatched larvae, and VtG levels were correlated using Person correlation (GRAPHPAD PRISM). Total hemolymph VtG was calculated using the total protein quantification and then averaged for spawned and non-spawned females. The value of $p < .05$ was accepted as significant.

The resultant allele sizes from the microsatellite analysis were compared using Arlequin v3.11 treating these Batches as separate populations. The analysis of molecular variance (AMOVA) was performed on the intra- and inter-population levels to determine relevant population statistics using the Arlequin default values.

3 | RESULTS

3.1 | Levels of total hemolymph protein

The concentrations of the total hemolymph protein are similar in spawned and non-spawned females at 47.9 ± 4.9 mg/ml hemolymph (*n* = 19) and 55.1 ± 9.2 mg/ml hemolymph (*n* = 17) respectively. These levels remain constant throughout the experimental period.

3.2 | Temperature effect on hemolymph VtG levels

As shown in Figure 1a, in spawned females, VtG levels fluctuate ranging from 1,534.1 ± 157.5 (*n* = 19) to 134.3 ± 17.2 μg VtG/ml hemolymph (*n* = 19), accounting for 3.2%–0.3% of the total hemolymph protein respectively. Vitellogenin levels of the spawned females at 21°C (*n* = 19), display two cyclic events: one between weeks −14 and −8 and the other between weeks −2 and +4. The VtG levels at weeks −13 and −11 were significantly higher than the basal level (dotted line) prior to spawning, the level dropped at the time of spawning, then increased significantly afterwards ($p < .05$).

In non-spawned females (Figure 1b), VtG levels are ranging from 992.5 ± 152.1 to 82.7 ± 22.8 μg VtG/ml hemolymph (*n* = 8), accounting for 1.8%–0.2% of the total hemolymph protein respectively.

At 11°C, all animals did not spawn and were kept consistent levels of VtG ranging from ~40–90 μg to 266.0 ± 154.2 μg/ml hemolymph (*n* = 9) throughout the 19 week experimental period (Figure 1c).

3.3 | Photoperiod effect on hemolymph VtG levels of spawned females

Vitellogenin levels for the 21°C females initially ranged from ~200 to 400 μg VtG/ml hemolymph for 5 weeks then increased to ~600–1,000 μg/ml hemolymph before spawning, after which VtG levels decreased to ~400 μg/ml hemolymph for the remainder of the sampling (Figure 2). Overall, VtG levels increased until spawning when they decreased temporarily and increased again after the spawning event. At 11°C, VtG levels at each photoperiod were

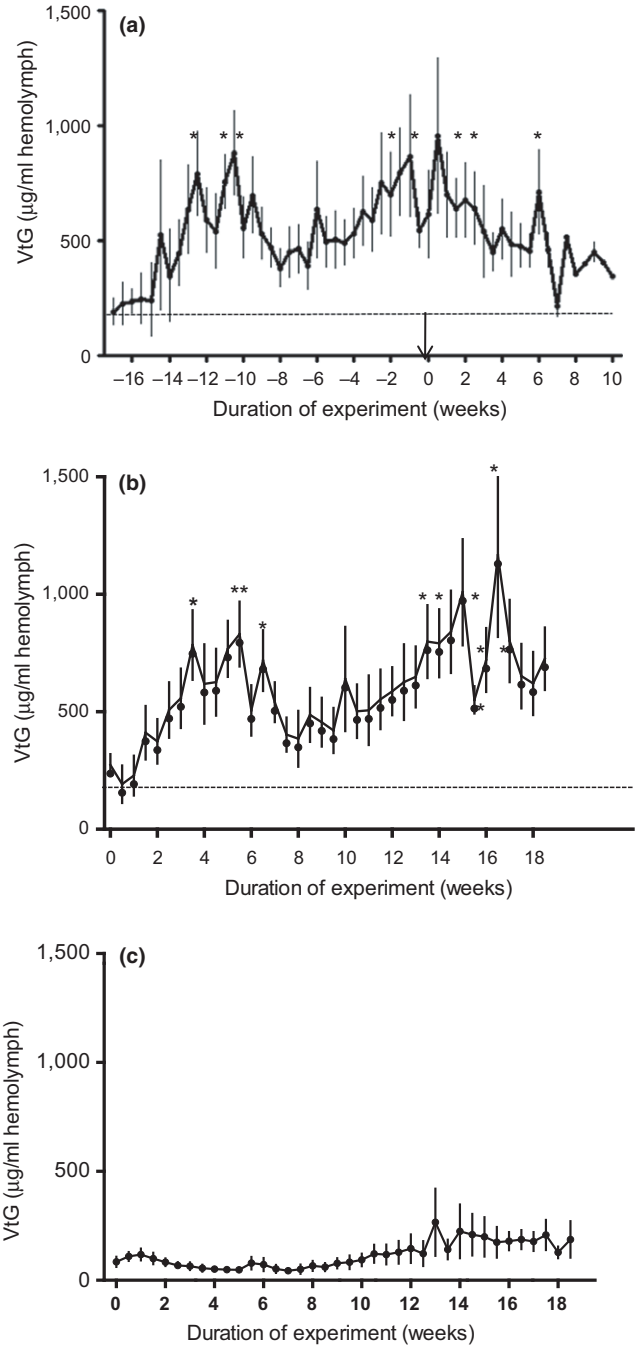

FIGURE 1 The effect of temperatures on vitellogenin (VtG) concentrations of females. (a) spawned at 21°C (*n* = 19); (b) non-spawned at 21°C (*n* = 8); and, (c) non-spawned at 11°C (*n* = 9) during the sampling dates show contrasting patterns. The results are presented as mean ± 1 *SE*. Dotted lines are marked at the lowest VtG concentrations in both a and b. The time of spawning is indicated by point 0. Statistical significance (Student's *t* test) is obtained from between the basal level and peak. *$p < .05$; **$p < .01$; ***$p < .001$

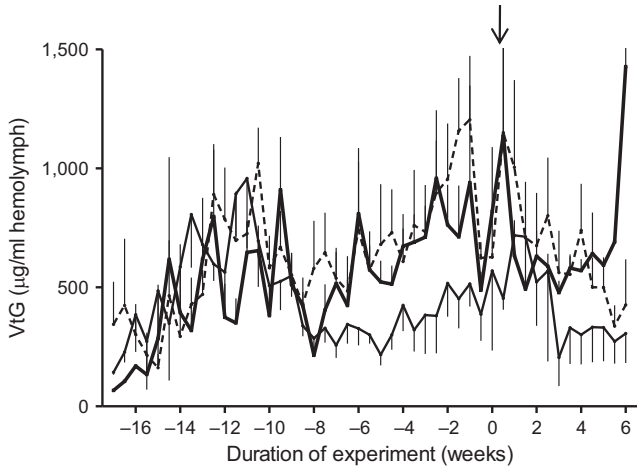

FIGURE 2 The effect of photoperiod on vitellogenin (VtG) concentrations of the spawned females. Vitellogenin levels are compared for photoperiods at 21°C. Vitellogenin levels are fluctuated between the photoperiods. The arrow at 0 indicates the spawning. Vitellogenin levels are presented as mean ± 1 *SE* with (1) 0L:24D (n = 6, dark solid line), (2) 8L:16D (n = 8, dotted line), and (3) 16L:8D (n = 5, thin solid line)

not determined due to lack of replicates that resulted from high mortality.

3.4 | Hemolymph VtG levels of multiple spawned animals

The data presented in Figure 3 was obtained from monitoring VtG levels during multiple spawning events of two animals kept at 21°C at 0L:24D (Figure 3a) and 16L:8D (Figure 3b). Both animals presented dynamic, fluctuating VtG levels. The VtG level of the animal presented in Figure 3a ranged from the lowest 202 μg/ml hemolymph at −9 weeks to the highest 1,864 μg/ml hemolymph at −6.5 weeks. The crab produced a total of 3.01 million larvae through three spawns (arrows; 1st = 1.58 millions; 2nd = 1.13 millions; 3rd = 0.39 millions). Figure 3b showed the lowest VtG of 72 μg/ml hemolymph at −5 weeks and the highest of 1,120 μg/ml hemolymph at 1.5 weeks, while producing a total of 7.91 million larvae in two spawns (1st = 1.91 millions; 2nd = 6.00 millions).

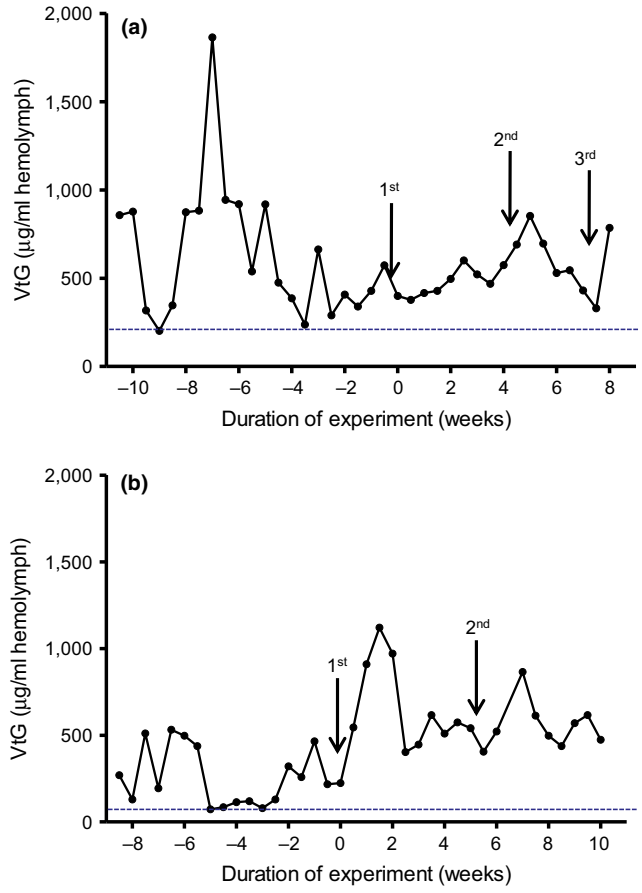

FIGURE 3 Vitellogenin (VtG) levels of two multiple spawned representative females kept at 21°C (a) at 24D:0L and (b) at 8D:16L during the sampling period. In each case, the 0 point indicates the first spawning event indicated by the first arrow while subsequent spawns were also denoted with arrows [Colour figure can be viewed at wileyonlinelibrary.com]

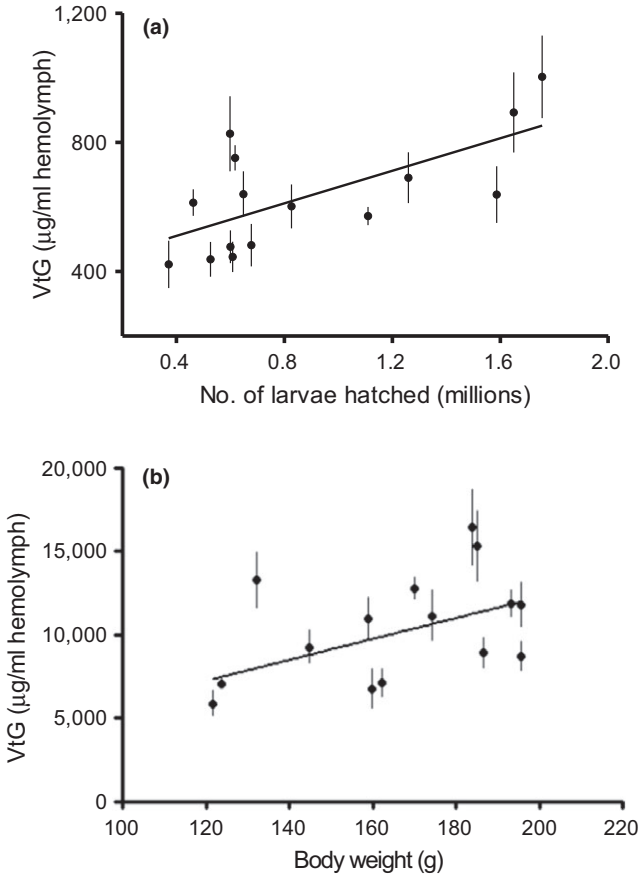

FIGURE 4 Vitellogenin (VtG) concentrations correlated to the number of larvae hatched and female size. (a) Female VtG levels correlate with the number of larvae hatched (r^2 = .45). (b) A positive correlation is present when assessing the total hemolymph volume (r^2 = .2). Vitellogenin concentrations were presented as mean ± 1 *SE* (n = 15)

3.5 | Hemolymph VtG levels versus number of larvae hatched and female weight

Vitellogenin concentrations that are averaged up to the point of spawning were positively correlated with the total larvae produced for that spawning event (r^2 = .45) (Figure 4a). Averaged VtG concentrations of animals with body weights (122–196 g) range from 420 to 1,003 μg/ml hemolymph. There was no correlation between the size of animals and the averaged VtG concentrations. However, assuming the total hemolymph volume is a minimum of 10% of the crab's body weight (Maynard, 1960), the total VtG per animal increased with body weight (Figure 4b). Therefore, heavier females produced more VtG, resulting in larger spawns.

3.6 | Microsatellite analysis of different batches of *Callinectes sapidus*

The microsatellite data showed large intra-population diversity within each batch combinations. In all batches, the number of observed alleles is larger than the sample size (Table 2). The level of heterozygosity was also high with 100% of samples in Batches 2 and 3 and 88% of the samples in Batch 1. For the data shown in Table 3, we included another batch of animals (Batch 3) that were obtained in April of the same year as Batch 2. A statistically significant relationship was noted between Batches 2 and 3, exclusive of Batch 1 ($p < .05$) with a low F_{ST} value of .018.

TABLE 2 The analysis of individual population statistics by the analysis of molecular variance (AMOVA)

Population statistics	Batch 1	Batch 2
Allele number	18	24
Observed heterogeneity	0.8824	1.0000
Allelic range	40	70
Sample size	17	41

"Allele number" lists the total unique product sizes for each group, "Observed heterogeneity" is listed as a proportion of heterozygous samples, "Allelic range" lists the difference in size between the largest and smallest products, and the sample size is the total number of unique isolates from each batch that gave measurable microsatellite data.

TABLE 3 The analysis of F_{ST} values for comparisons of the three batches using analysis of molecular variance (AMOVA)

F_{ST} values (110 permutations)	Batch 1	Batch 2	Batch 3
Batch 1	0		
Batch 2	0.00653	0	
Batch 3	−0.00573	0.01808*	0

The F_{ST} values for comparisons of the two groups using AMOVA are presented in this table. An * represents a significantly different population with p = .01256 ± .0017. The percentage of variation among these populations is 0.60%, while the within population percentage is 99.4% (Batch 1: n = 19; Batch 2: n = 17; and Batch 3: n = 42).

4 | DISCUSSION

This study determined the influence of environmental conditions on VtG levels of *C. sapidus*. In general, these results show continuous presence of VtG over spawning activity, while the levels increase prior to and after spawning with a brief pause, occurring during spawning.

The protein levels in the hemolymph of crustaceans typically describe their physiological status, specifically regarding moulting. The comparison of the percentage of VtG in the total hemolymph protein at the highest and lowest values for spawned versus non-spawned females show that the VtG levels are higher for spawned females with 0.26%–3.16% of the total hemolymph protein, whereas non-spawned crabs range from 0.15% to 1.80%. The high levels of hemolymph VtG of spawned females indicate continuously producing VtG to be uptaken by the oocytes even over spawning events. The lower VtG levels in non-spawned females imply a possibly slow vitellogenic activity including VtG synthesis, transport and uptake even at 21°C. These results suggest that spawning itself may play a positive role in next round of ovarian development. Additionally, all non-spawned females during the experimental period did produce spawning in 4–8 weeks after ending the experiment.

4.1 | Temperature and photoperiod on hemolymph VtG levels

In keeping with previous findings (Bembe et al., 2017), temperature affects the levels of hemolymph VtG in *C. sapidus*. The animals exposed to higher temperature at 21°C carry higher levels of hemolymph VtG than those at 11°C. The VtG levels are continuously fluctuating prior to and after spawning in *C. sapidus*, as reported (Lee & Puppione, 1988). However, VtG is always present in the hemolymph of our animals, whereas the absence of VtG reported in the earlier study varied from 0 ± 0.00 to 4.1 ± 2.1 mg/ml hemolymph of the animals at ovarian stages 1–8 (Lee & Puppione, 1988). This difference may be due to the fact that our animals were in 3–4 ovarian stages. In this study, the VtG levels slightly increase prior to the spawning, then decrease during the spawning, and increase afterwards with levels consistently remaining above ~200 μg VtG/ml hemolymph (Figure 1a). A similar pattern to this phenomenon is noted in *H. americanus* (Byard & Aiken, 1984; Nelson, 1986), *M. nipponense* (Okumura et al., 1992) and *M. rosenbergii* (Derelle et al., 1986; Okumura & Aida, 2001).

A positive relationship between size and number of larvae hatched intuitively suggests that larger size of females may produce greater amounts of VtG and indeed larger females had higher averaged VtG levels (Figure 4a). Interestingly, the concentration of VtG/ml seems constant regardless of size of animals, indicating the importance of hemolymph volume and the size of animal. Heavier females have a greater hemolymph volume and carry more VtG to produce larger spawns. This observation supports earlier findings on a positive relationship between the body weight of females and

larval production (Bembe et al., 2017) as well as fecundity and carapace width (Hines, 1982; Prager, McConaugha, Jones, & Geer, 1990).

The spawning of *C. sapidus* was clearly influenced by temperature in this study as in the natural population. In the Chesapeake Bay, *C. sapidus* spawns in water temperatures ranging from 21 to 27°C from July to September (Sandoz & Rogers, 1944). Similarly, areas such as Florida experience spawning at these temperatures from April to November (Hines et al., 2003). In the Chesapeake Bay, crabs produce 1–3 spawns in a 3 month season, whereas in Florida the warmer season is longer allowing for up to 7 spawns (Hines et al., 2003). The constant 21°C during the 19 weeks (4.75 months) of this experiment allowed for VtG to be continuously produced and taken up resulting in 1–3 spawns per female.

At 11°C the females showed low levels of hemolymph VtG during the sampling period, resulting in no spawns and also high mortality (Bembe et al., 2017). In *H. americanus*, VtG levels range from zero to very low levels during the winter season (Byard & Aiken, 1984). The low levels observed in *C. sapidus* are in accordance with the reduced metabolic activity that occurs during overwintering periods in the wild (Churchill, 1917; Havens & McConaugha, 1990; Van Engel, 1958). During the overwintering period, animals are energetically and behaviourally arrested and will become active once exposed to warmer temperatures.

Photoperiod does affect the reproductive nature of the female *C. sapidus*. Overall, VtG levels reflected the frequency of spawning and were associated with the length of darkness. Females at 21°C in 0L:24D and 8L:16D produced more VtG than 16L:8D, which supports the result of increased spawning at 21°C, 0L:24D and 8L:16D over 16L:8D (Bembe et al., 2017).

It seems that monitoring hemolymph VtG levels appears to be an ideal way for evaluating the ovarian development without sacrificing the animals. Certainly high VtG levels in hemolymph are positively associated with spawning activity in general. However, hemolymph VtG levels that constantly fluctuate during the ovarian development, together with individual variations may allow for predicting the timing and frequency of spawning not precise, but within 2–4 week time frame.

4.2 | Genetic diversity

An earlier study on genetic analysis of juvenile *C. sapidus* caught from the wild showed a large amount of genetic variation, as the mitochondrial DNA of the Chesapeake Bay population is genetically diversified with a haplotype diversity of >0.7, resulting in a lack of common haplotypes (Feng et al., 2017). The population of *C. sapidus* is suggested to be annually re-structured through a new recruitment of juveniles.

The population dynamics within a year was examined using another batch of animals (Batch 3) that were caught in April of the same year as Batch 2 (November, 2007). The data in Tables 2 and 3 were obtained from only one marker. This is uncommon for microsatellite datasets, but in this case a significant amount of observed genetic diversity arises from a large allelic range, with up to 70 bp differences observed between the largest and smallest repeat sets in Batch 3. The F_{ST} value between Batches 2 and 3 was low (.02) which is likely due to the large intra-population diversity, which accounts for 99.4% of the observed variation. Batches 2 and 3 were obtained during different seasons (spring and fall) in the same year and were closely related in this genetic analysis. This implies that the recruitment of these animals that migrated down to the mouth of the Bay in different seasons, might be related. Those that were caught in different years varied from each other, supporting the previous finding of a high turn-over of *C. sapidus* population in the Chesapeake Bay.

A great degree of individual variation in VtG levels in different batches was observed with several reproductively inactive animals during the experimental period. This individual variation may be due to the genetic variation in the wild female population. Determining the relationship between the genetic diversity of each of these animals and its reproductive activity is an avenue of further study. Specifically, it remains to be determined whether the length of the microsatellites located in the MIH gene is performing a dual function of moult and vitellogenesis regulation in *C. sapidus* (Chung, Zmora, Tsutsui, & Katayama, 2010; Zmora, Sagi, Zohar, & Chung, 2009; Zmora, Trant, Zohar, & Chung, 2009) and can be directly linked to the reproductive performance of females.

5 | CONCLUSION

Females in warmer temperatures (21°C) have greater VtG levels than those in colder temperatures (11°C), while at 21°C, longer darkness (16–24D) have higher VtG levels than longer light (16–24L). Ovarian development including VtG synthesis, transport and uptake, is a continuous process in which the rate of each step may be differentially regulated causing a constant change in the hemolymph VtG levels of reproductively active and even inactive females. Interestingly, the great genetic diversity of different batches collected in the Chesapeake Bay requires further work on a possible connection between growth and reproductive performance that may be regulated by MIH.

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