Compared the effects of mTOR signaling pathway on molting gland in two crustaceans species: *Gecarcinus lateralis*, with *Carcinus maenas*

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Abstract:

The molting cycle in decapod crustaceans is controlled by the X-organ/sinus gland complex in the eyestalks (ES). The complex secretes molt inhibiting hormone (MIH), a neuropeptide produced in the eyestalk ganglia, inhibiting YO ecdysteroidogenesis. In most decapods molting was induced by eyestalk ablation (ESA), or autotomy of 5 or more walking legs (Multiple Leg Autotomy, MLA). However, the green shore crab C. maenus (both color morphs) is refractory to ESA and MLA. The mechanistic Target of Rapamycin (mTOR) pathway is highly conserved among all metazoans; it functions as a nutrient sensor for cellular growth and is up-regulated in mammalian cancers. cDNAs encoding mTOR components (mTOR; Ras homolog expressed in brain, Rheb; protein kinase B, Akt; and p70 S6 kinase, S6K) were cloned from blackback land crab, Gecarcinus lateralis, and green shore crab, Carcinus maenas. The purpose of this study was to quantify and compare the effects of molt manipulation (ESA and MLA), on mTOR pathway components (mTOR, Rheb, Akt, and S6K), in G. lateralis and C. maenas by qPCR. mTOR, which controls global translation of mRNA into protein, appears to be involved in YO activation. mTOR, Rheb, Akt and S6k were expressed in crustacean tissues including YO. Rapamycin, an mTOR inhibitor, is a potent inhibiter of YO edysteroidogenesis. YO activation is required for transition to the committed state, as indicated by the prolonged effect of rapamycin on ES-ablated animals. We hypothesize that up-regulation of mTOR signaling is necessary for YO hypertrophy and increased ecdysteroidogenesis during premolt. qPCR data indicate that ESA activates mTOR signaling in the G. lateralis YO, but not in the C. maenas YO.

Key words: *mTOR, Gene Expression, blackback land crab, Crustacean, Ecdysteroid, Molting, Y-organ, Eyestalk Ablation, mRNA.*

مقارنة تأثيرات مسار تخليق البروتين الـmTOR على غدد الانسلاخ في نوعين من القشريات: *Gecarcinus lateralis* ، مع *Carcinus maenas*

المستخلص:

يتم التحكم في عملية إنسلاخ القشريات عن طريق مجمع غدد الجيوب الأنفية والتي تفرز هرمون مثبط للانسلاخ (MIH) وهو ببتيد عصبي ينتج في العقد العصبية في العين يعمل على تثبيط غدد الانسلاخ (YO) من إفراز هرمون الاكيدستيرويد. وفي معظم القشريات يتم حث عملية الانسلاخ بطريقتين إما باستئصال ساق العين

(ESA) أو قطع ذاتي لخمسة أو أكثر من أرجل المشي (MLA)، وهذا الامر لا ينطبق على سرطانات البحر الخضراء فهي لا تستجيب لعمليتي الحث المذكورة. إن مسار تخليق البروتين في الخلية متشابه بدرجة كبيرة في كل الكائنات الحية عديدة الخلايا وهو يستخدم كحساس لعوامل النمو في الخلية ومنظم لأمراض السرطان في الثدييات. لقد تم نسخ جينات مسار الـ (mTOR) في سرطان البحر الأسود *G. lateralis* وسرطان البحر الأخضر *C. maenas* وهي (mTOR, Rheb, Akt & S6k). الغرض من هذه الدراسة تحديد ومقارنة تأثير عمليات حث الانسلاخ (ESA & MLA) على جينات مسار تخليق البروتين لـ mTOR (mTOR, Rheb, Akt & S6k) في الـ *G. lateralis* والـ *C. maena* بواسطة الـqPCR. يتحكم مسار تخليق البروتين الـmTOR في عمليات ترجمة الـmRNA إلى بروتين وهو أيضاً يشارك في نشاط غدد الإنسلاخ حيث وجد التعبير الجيني لكل جينات الـmTOR في أنسجة القشريات بما فيها غدد الانسلاخ ويعتبر الـmTOR مثبط قوي لإفراز الأكيدستيرود هرمون من غدد الانسلاخ، وعملية تنشيط غدد الانسلاخ يتطلب الانتقال إلى عدة مراحل منها مرحلة الإفراز، نحن نفترض أن زيادة تنظيم مسار الـmTOR ضروري لزيادة حجم غدة الانسلاخ وزيادة إفراز هرمون الإكيدستيرويد كما تشير لذلك نتائج عمليات حث الانسلاخ في الـ *G. lateralis* وليس الـ *C. maenas*.

الكلمات المفتاحية: *القشريات، الرنا الرسول، غدة الإنسلاخ، الاكيدستروييد، غدد الجيوب الأنفية، التعبير الجيني.*

Introduction:

The molting gland, is the source of steroid hormone production and consequent molt cycle regulation, the molting cycle in decapod crustaceans is mainly controlled by X-organ/sinus gland (XO/SG) complex and the Y-organ (YO). Molting-inhibiting hormone (MIH), is produced in XO/SG complex, and regulates ecdysteroidogenesis in the YO (Chang and Mykles, 2011; Hopkins, 2012; Webster, 2015).

In most decapod crustaceans molting can be induced by eyestalk ablation (ESA) or by autotomy of at least 5 walking legs (multiple leg autotomy or MLA). ESA removes the primary source of MIH and results in an immediate activation of the YO and an increase in hemolymph ecdysteroid titers within 1 day. However, the green shore crab *C. maenus* (both color morphs) is refractory to ESA and MLA (Covi et al., 2010 and MacLea et al., 2012).

mTOR is a protein kinase that controls protein synthesis. It functions as the major sensor for cellular growth regulation by nutrients, cellular energy status, oxygen level, and growth factors (Fig. 1). mTOR phosphorylates S6 kinase (S6K) and eIF4E-binding protein, which stimulates global translation of mRNA into protein. Rheb and Akt activate mTOR YO ecdysteroidogenesis is inhibited by cycloheximide, an inhibitor of translation (Fig. 2), but not actinomycin D, an inhibitor of transcription (Proud, 2009 and Laplante et al., 2012).

The YO goes through four physiological states during the molt cycle that are mediated by endocrine and autocrine factors. A reduction in MIH triggers the transition from the basal state in intermolt to the activated state in early premolt; a putative TGFβ factor triggers the transition from the activated state to the committed state in mid premolt; and high ecdysteroids trigger the transition from the committed

state to the repressed state in late premolt (Abuhagr et al., 2016).

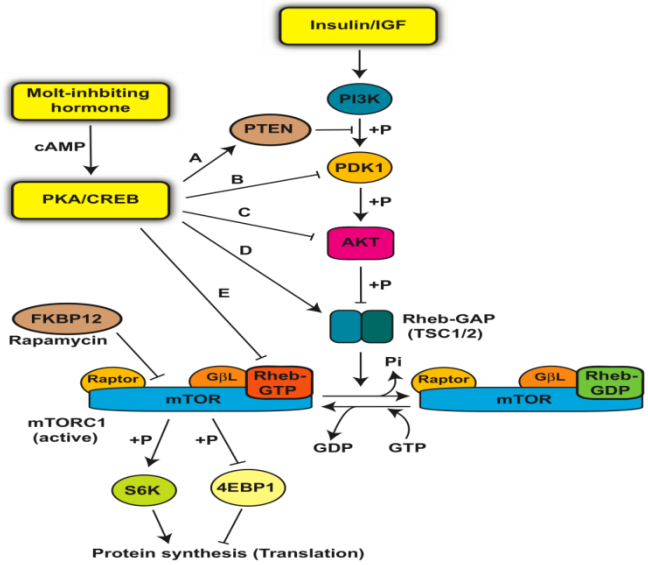

Figure 1. Proposed regulation of mTORC signaling pathway. Rheb-GAP (TSC1/2) inactivates mTORC1 by promoting the hydrolysis of GTP to GDP. Rapamycin inhibits mTORC1 via binding to FKBP12. Insulin/IGF signaling (PI3K, PDK1, & Akt) activates mTORC1 by inhibiting Rheb-GAP. We hypothesize that Mstn/Smad signaling inhibits mTORC1 by altering expression and subsequent phosphorylation of insulin/IGF signaling components, either through up-regulation of PTEN (A) and/or Rheb-GAP (D), down-regulation of PDK1 (B), Akt (C), and/or Rheb (E), or a combination of any or all.

In most decapods, including *G. lateralis*, molting is induced by ESA or MLA. YO ecdysteroidogenesis is inhibited by cycloheximide, an inhibitor of translation, but not actinomycin D, an inhibitor of transcription (Webster, 1993).

The YO is a dynamic organ that changes over the molt cycle. MIH suppresses ecdysteroidogenesis by the YO during intermolt, but the YO becomes refractory to MIH by late premol (Covi et al., 2010 and MacLea et al., 2012). There is no reduction in MIH receptors during intermolt or premolt (Webster, 1993), which suggests that the desensitization of MIH signaling is downstream from the receptor, possibly through changes in the levels and activities of phosphodiesterases (PDEs) and NO/cGMP signaling components. Increased PDE activity contributes to the reduced response to MIH by keeping intracellular cyclic nucleotides low (Chang and Mykles, 2011; and Nakatsuji et al., 2009). At the end of premolt there is a precipitous drop in hemolymph ecdysteroids within a few days of ecdysis (Skinner, 1985 and Mykles, 2011). This drop appears to determine the timing of ecdysis, as artificially elevated ecdysteroid during late premolt delays ecdysis (Chang and Mykles, 2011). It is the result of two processes: an increase in ecdysteroid excretion and a decrease in YO ecdysteroid production. 20E

inhibits YO ecdysteroidogenesis when injected into crayfish (Dell et al., 1999). Both treatments produce significant reductions in ecdysteroidogenesis within 1 h, suggesting a non-genomic response mediated by G protein-coupled and/or membrane-associated ecdysteroid receptors (Srivastava et al., 2005 and Schlattner et al., 2006). This inhibition lasts at least 24 h after a single 20E injection (Dell et al., 1999), which suggests that ecdysteroid may also affect gene expression.

mTOR is a protein kinase highly conserved among all metazoans; that controls protein synthesis. It functions as the major sensor for cellular growth regulation by nutrients, cellular energy status, oxygen level, and growth factors (Proud, 2009 and Laplante et al., 2012).

The central hypothesis is that YO ecdysteroidogenesis requires up-regulation of mTOR. MIH suppresses the mTOR pathway. YO commitment requires a TGFb factor acting through Activin receptor/Smad signaling, resulting in sustained mTOR activation, up-regulation of ecdysteroid biosynthetic enzymes, and down-regulation of MIH signaling. The specific aim to determine the effects of ESA and MLA on molting and YO gene expression in *G. lateralis* and *C. maenas*.

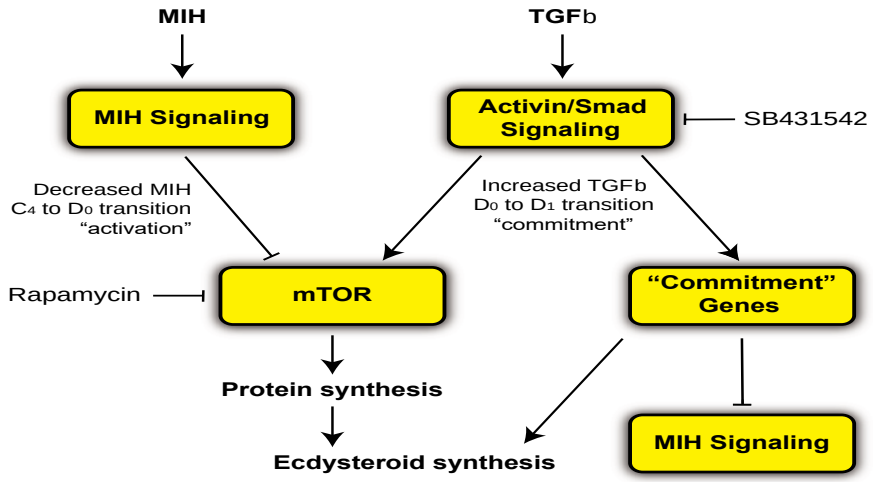

Figure 2. Signaling pathways controlling YO ecdysteroid synthesis. MIH inhibits YO during intermolt. At mid premolt a putative TGFβ factor produced by the activated YO stimulates mTOR and "commitment" genes that inhibit MIH signaling and stimulate ecdysteroid biosynthetic enzymes. Rapamycin inhibits mTOR and SB431542 inhibits TGFβ.

Materials and Methods:

Adult *G. lateralis* were collected in the Dominican Republic and shipped via commercial air cargo to Colorado, USA. Animals were maintained at 27 ºC in 75-90% relative humidity with intermolt individuals kept in communal plastic cages lined with aspen bedding wetted with 5 p.p.t. Instant Ocean (Aquarium Systems, Mentor, OH). The crab environmental chamber was maintained in 12 h: 12 h light: dark cycle

with twice-weekly animal feedings of carrots, iceberg lettuce, and raisins (Covi et al., 2010). These crabs molt approximately once a year. Molting is easily manipulated in *G. lateralis* by ES ablation (ESA). ESA is an effective and convenient method. The major advantage is that ESA provides a precise reference point for YO activation. In *G. lateralis*, hemolymph titers increase by 1 day post-ESA (Lee et al., 2007). Animals enter premolt immediately, but do not successfully complete ecdysis (Covi et al., 2010).

MLA experiment:

G. lateralis. Animals were divided into three premolt stages (early premolt, R ~10; mid-premolt, R ~15; and late premolt, R ~22) and two postmolt stages (2 days and 10 days postmolt). Hemolymph samples were collected for ecdysteroid titers using ELISA and YOs were harvested for qPCR. *C. maenas* were refractory to MLA. Instead, animals at various molt stages (intermolt, early premolt, late premolt and postmolt) were collected during the spring molting season. Hemolymph samples were collected for measuring ecdysteroid and YOs were harvested for qPCR for reviews, see (MacLea et al., 2012).

ESA experiment:

In *G. lateralis*, hemolymph titers increase by 1 day post-ESA (Lee et al., 2007). Animals enter premolt immediately, but do not successfully complete ecdysis.

Intact intermolt *G. lateralis* were ES-ablated. Hemolymph samples were taken and YOs were harvested at 0, 1, 3, 7, and 14 days post-ESA. Hemolymph ecdysteroid was quantified by ELISA (Chang and Mykles, 2011). YOs were harvested at 0, 1, 3, 7, and 14 days post-ESA.

C. maenas (both red and green color morphs) were ES-ablated. Hemolymph samples were taken and YOs were harvested at 0, 7, and 14 days post-ESA (Covi et al., 2010).

RNA was isolated, DNase-treated, and reversed transcribed into cDNA. Gl-EF2, Gl-mTOR, Gl-Rheb Gl-Akt and Gl-S6k, mRNA levels were quantified with Roche Light Cycler 480 using sequence-specific primers. Tissues were flash-frozen in liquid nitrogen and stored at -80 ˚C. Total RNA was isolated from crab tissues using TRIzol reagent (Life Technologies, Carlsbad, CA) as described previously (Covi et al., 2010). A LightCycler 480 thermal cycler (Roche Applied Science, Indianapolis, IN) was used to quantify levels of EF2, mTOR, Rheb, Akt and S6k mRNAs for *G. lateralis*. Reactions consisted of 1 µl first strand cDNA or standard, 5 µl 2× SYBR Green I Master mix (Roche Applied Science), 0.5 µl each of 10 mM forward and reverse primers (Table 1), and 3 µl nuclease-free water. Total RNA in the cDNA synthesis reaction were calculated based on the standard curve and the calculated molecular weight of dsDNA products.

Table 1. Oligonucleotide primers used in the expression analysis (qPCR) of mTOR signaling components from both *G. lateralis* and *C. maenas*.

Abbreviations: Gl, *G. lateralis*; F, forward; R, reverse; EF2, elongation factor 2; mTOR, mechanistic Target of Rapamycin; Rheb, Ras homolog expressed in brain; Akt, protein kinase B; S6k, p70 S6 kinase.

Primer	Sequence (5'-3')	Product Size (bp)
Gl-EF2 F1 Gl-EF2 R1	TTCTATGCCTTTGGCCGTGTCTTCTC ATGGTGCCCGTCTTAACCA	227
Gl-mTOR F2 Gl-mTOR R2	AGAAGATCCTGCTGAACATCGAG AGGAGGGACTCTTGAACCACAG	159
Gl-Rheb F1 Gl-Rheb R1	TTTGTGGACAGCTATGATCCC AAGATGCTATACTCATCCTGACC	119
Gl-Akt F2 Gl-Akt R1	AACTCAAGTACTCCAGCGATGATG GGTTGCTACTCTTTTCACGACAGA	156
Gl-s6k F2 Gl-s6k R1	GGACATGTGAAGCTCACAGACTTT TTCCCCTTCAGGATCTTCTCTATG	239
Cm-EF2 F1 Cm-EF2 R1	CCATCAAGAGCTCCGACAATGAGCG CATTTCGGCACGGTACTTCTGAGCG	278
Cm-mTOR F2 Cm-mTOR R2	CATCCCTCAAACCTCATGCT CACCCACCACAGAACGCTTT	319
Cm-Rheb F2 Cm-Rheb R2	ATGGGCAAAGTCACAGTTCC GTCAGGAAGATGGTGGCAAT	218
Cm-Akt F1 Cm-Akt R1	GTGAAGCAATGCCAGATCCT CGGGTGTATCATCATCATCG	259
Cm-s6k F2 Cm-s6k R2	TCTCCGTCATCTGAGCCGCT GTACATGGCACCCGAGATCC	258

Statistical analyses:

Statistical analysis was performed using JMP 5.1.2, 6.0.0, or 8.0.2 (SAS Institute, Cary, NC). Means for different developmental stages and treatments were compared using analysis of variance (ANOVA). All data not plotted as individual points are represented as mean ± 1 S.E. and the level of significance for the all the data analyses was set at $\alpha = 0.05$. All qPCR data was log transformed to reduce the variance of the mean. The data were performed using Excel 2010 (Microsoft, Redmond, WA) and JMP. Excel 2013.

Results and Discussion:

In *G. lateralis* hemolymph ecdysteroid titers are a function of YO ecdysteroid synthetic activity (Mykles, 2011 and Chang and Mykles, 2011). ESA caused an increase in hemolymph ecdysteroid titer in starting at 1 day post-ESA (Fig. 3A). At 7-14 days post-ESA the animal transitions from early premolt to late premolt (Covi et al., 2010), resulting in a large increase in hemolymph ecdysteroid titer from 7 days post-ESA to 14 days post-ESA. ES-ablated animals showed a significant increase in hemolymph ecdysteroid titers at Days 1, 3, 7 and 14 compared with intact at Day 0 (Fig. 3A).

The expression of *Gl-EF2*, *Gl-mTOR*, *Gl-Rheb*, *Gl-Akt* and *Gl-S6k* were significantly increased by 1 day post-ESA (Fig. 3B-F).

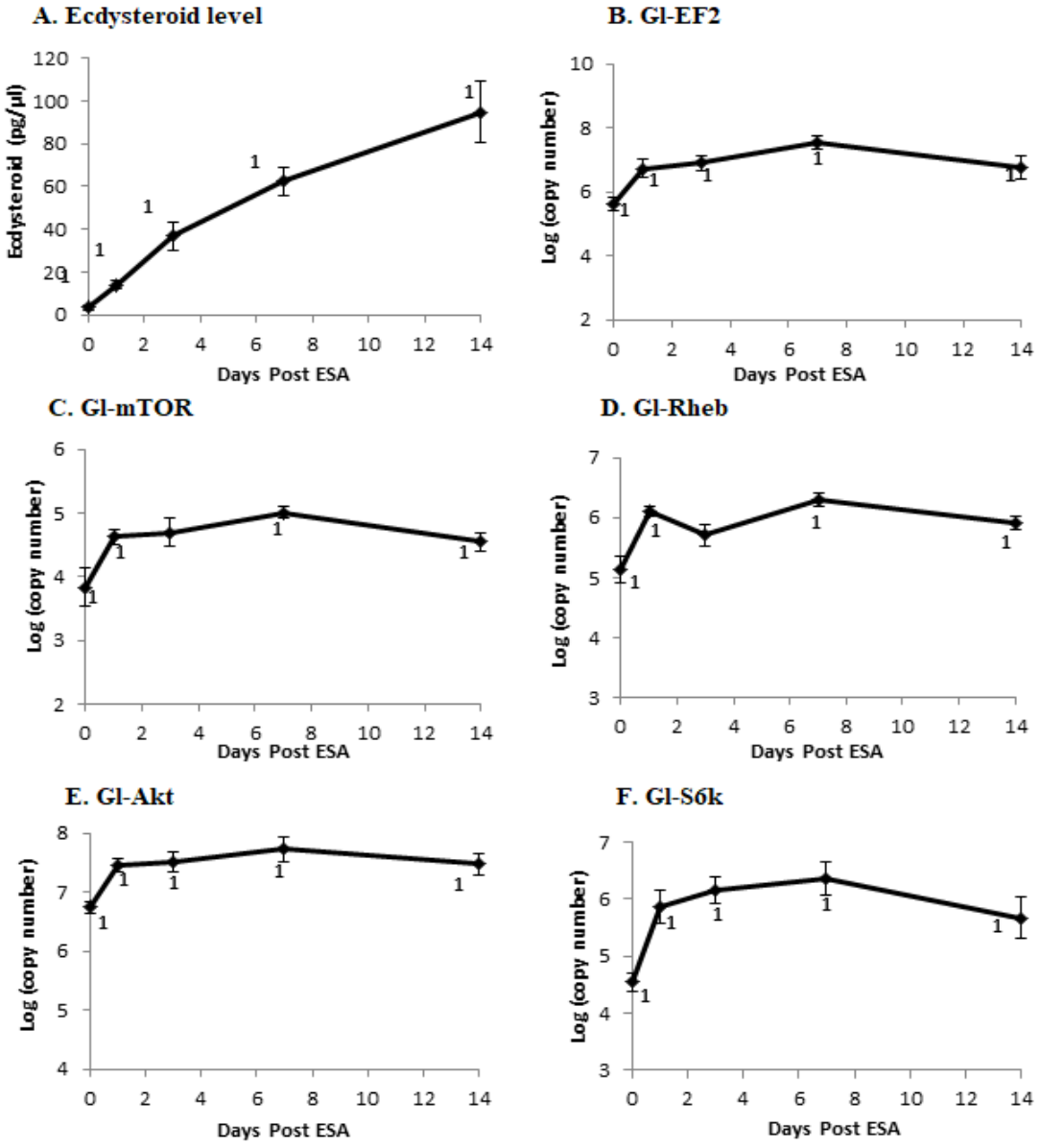

Figure 3. Effects of ESA on hemolymph ecdysteroid titer (A) and YO expression of Gl-EF2 and mTOR signaling components (B-F) in *G. lateralis in vivo*. Hemolymph and YOs tissues were collected from intact (Day 0) and ES-ablated animals (1, 3, 7 and 14 days post-ESA). Data are presented as mean ± 1 S.E. (sample size for each treatment: Day 0, n = 8; Days 1, 3, and 7, n = 5; Day 14, n = 7). Means within treatments that were significantly different from each other have the same number.

The expression levels between intact and ESA treatments converged at 14 days post-ESA for all five genes (Fig. 3B-F). *Gl-EF2* mRNA level showed a significant increase from 1 day post-ESA to 3 and 7 days post-ESA (Fig. 3B). The means of the ESA animals were significantly greater than the means of the intact animals at 3 and 7 days post-ESA (Fig. 3B).

Expression of *Gl-mTOR* mRNA increased significantly at 3 and 7 days post-ESA, when compared with day 0 (Fig. 3C). The means of the intact and experimental treatments were significantly different at 3 and 7 days post-ESA (Fig. 3C). *Gl-Rheb* mRNA level increased significantly at 1 and 7 days post-ESA (Fig. 3D). However, there were no significant differences in the means between intact and experimental treatments at 3 days post-ESA (Fig. 3D). There was as small, but significant, increase in *Gl-Akt* expression in experimental animals at 3 and 7 days post-ESA (Fig. 3E). In the ESA treatments, there were significant increases in *Gl-Akt* mRNA levels at 3 and 7 days post-ESA and the difference of the means between intact and experimental treatments at Days 1, 3, and 7 days post-ESA were statistically significant (Fig. 3E). *Gl-S6k* mRNA level in experimental animals increased significantly from 1 to 7 days post-ESA (Fig. 3F). There were significant differences between the means of the intact and experimental treatments at 1, 3 and 7 days post-ESA (Fig. 3F).

In the land crabs *G. lateralis*, molting is induced by eyestalk ablation (ESA). mTOR, which controls translation of mRNA into protein, appears to be involved in YO activation in early premolt, as rapamycin inhibits YO ecdysteroidogenesis *in vitro* and *in vivo*. At the activated to committed state transition, the animal becomes committed to molt, as the YO is less sensitive to MIH and premolt is not suspended by LBA. These data are consistent with the hypothesis that the increase in protein synthesis is dependent on increased ecdysteroid levels in circulating hemolymph (Mykles and Skinner, 1982; Mykles, 1999 and Abuhagr et al., 2015). How exactly ecdysteroid titers are mediating this process is a key area of investigation. It is clear from the analysis that mTOR components transcript levels increase with increasing ecdysteroid levels in the hemolymph in ESA (Matsakas and Patel, 2009). This observed upregulation of mTOR activity and its well understood downstream effectors of p70S6kinase and 4E-BP1, important for increasing translation of mRNA into protein (Hietakangas and Cohen, 2009).

Adult *C. maenas* are refractory to molt induction by ESA or MLA (Fig. 4), gene expression in the YOs of animals (green morphs) undergoing natural molts was quantified. Crabs were collected during the spring molting season in Bodega harbor. The hemolymph ecdysteroid titers showed the characteristic pattern over the molt cycle: low levels during intermolt, increasing levels during premolt, and lowest levels during postmolt (Fig. 4A). The YOs from these same animals were used to quantify *Cm-mTOR*, *Cm-Rheb*, *Cm-Akt*, *Cm-S6k*, and *Cm-EF2* expression. The postmolt stage was not quantified, as the RNA concentrations obtained from YOs from postmolt animals were too low for cDNA synthesis. Unlike *G. lateralis*, there was no effect of molting on the expression of *Cm-EF2* and mTOR signaling components. There were no significant differences in the means of the five mRNAs between intermolt, early premolt, and late premolt stages (Fig. 4B).

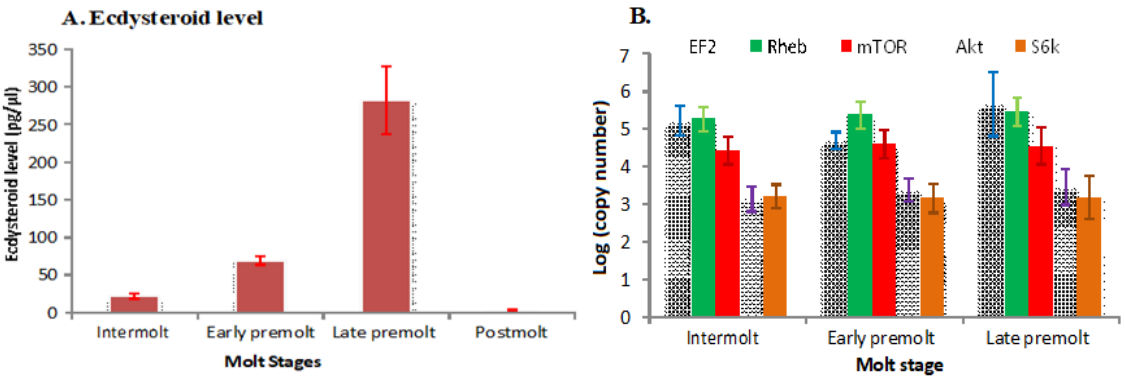

Figure 4. Effects of molting on hemolymph ecdysteroid titers (A) and YO expression of *Cm-EF2* and mTOR components (B) in *C. maenas*. Hemolymph ecdysteroid levels were quantified by ELISA. *Cm-mTOR*, *Cm-Rheb*, *Cm-Akt*, *Cm-S6k*, and *Cm-EF2* mRNA levels at intermolt, early premolt, and late premolt stages were quantified by real-time PCR (see Materials and methods). Data are presented as mean ± 1 S.E. (intermolt, n = 6; early premolt, n = 12; late premolt, n = 6; and postmolt, n = 8). There were no significant differences in the means for all five genes at all the molt stages.

In *C. maenas*, ESA had little effect on hemolymph ecdysteroid titer and no effect on expression of mTOR signaling components. There were no differences between green and red morphs (Fig. 5). Hemolymph ecdysteroid levels remained low, although there was a 64 significant decrease in hemolymph ecdysteroid level at 7 days and 14 days post-ESA in both color morphs (Fig. 5A). There was no significant effect of ESA on the expression of Cm-mTOR, Cm-Rheb, Cm-Akt, Cm-S6k and Cm-EF2 in red morphs (Fig. 5B) and green morphs (Fig. 5C).

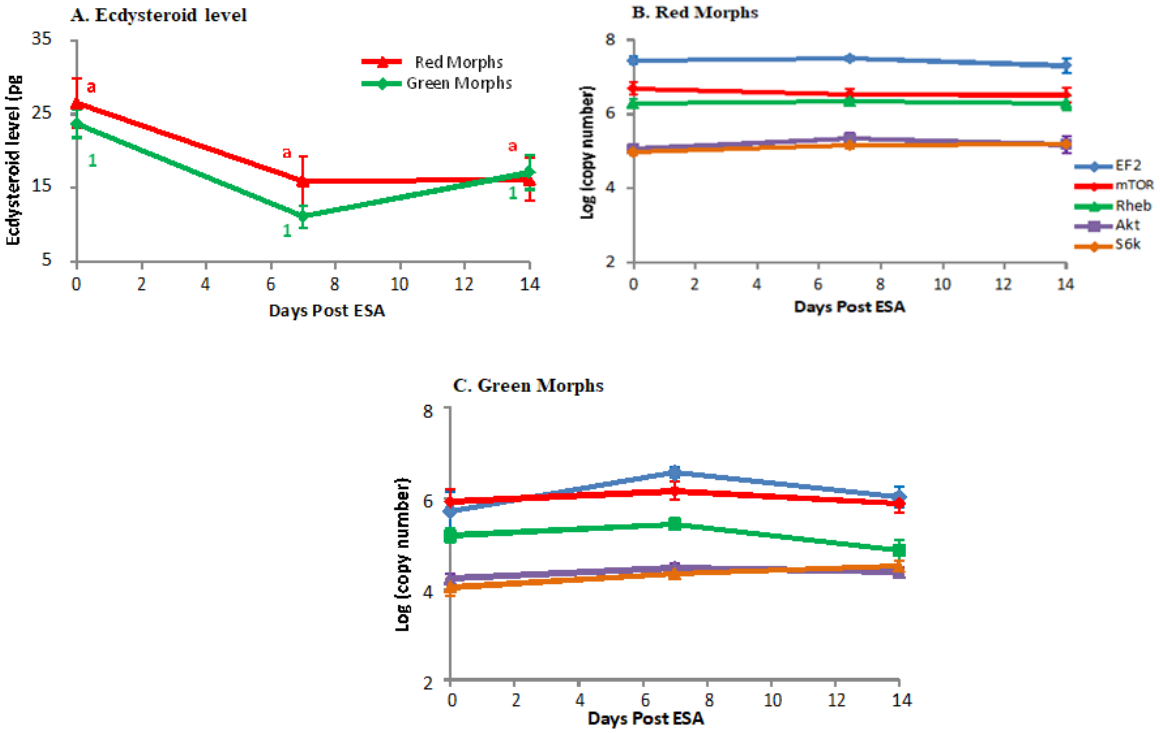

Figure 5. Effects of ESA on hemolymph ecdysteroid titer (A) and YO expression of *Cm-EF2* and mTOR components in *C. maenas*. Intermolt red (B) and green (C) morphs were ES-ablated at Day 0. Hemolymph and YOs tissues were collected from intact (Day 0) and at 7 days and 14 days post-ESA (see Materials and methods). Means of ESA animals that were significantly different from intact control (Day 0) are indicated by "1" for green morphs and "a" for red morphs (A). There was no significant effect of ESA on expression of the five genes.

Green crab *C. maenas* and land crab *G. lateralis* differed in their response to ESA. *G. lateralis* soon entered premolt and proceed to ecdysis, although most do not successfully molt; this corresponds to increasing ecdysteroid level that reached a peak by the end of late premolt (Fig. 4A) (Covi et al., 2010). ESA did not have any effect on *C. maenus* mTOR signaling components expression nor was there any increase in hemolymph ecdysteroid levels (Fig. 5). These data suggest that both red and green morphs are resistant to ESA (Abuhagr et al., 2014).

Adult green crabs (green morphs) captured during late winter and early spring underwent spontaneous molting. YOs were harvested from animals at intermolt, early premolt, late premolt and postmolt stages. Molt stage had no significant effect on the expression of *Cm-mTOR*, *Cm-Rheb*, *Cm-Akt*, *Cm-S6k* and *Cm-EF2* (Fig. 4B), suggesting that increased expression of mTOR signaling components is not required for YO ecdysteroidogenesis in *C. maenas*.

Conclusion:

In the blackback land crabs *Gecarcinus lateralis*, molting was induced by eyestalk ablation (ESA) or autotomy of 5 or more walking legs (multiple leg autotomy MLA). mTOR, which controls translation of mRNA into protein, appears to be involved in YO activation in early premolt. Quantitative PCR data indicated up-regulation of Gl-EF2 and mTOR that may reflect an increase in protein synthetic capacity in the premolt YO.

By contrast, *C. maenas* were refractory to ESA. In green shore crab, Unlike *G. lateralis*, molting had no effect on expression of *Cm-mTOR*, *Cm-Rheb*, *Cm-Akt*, and *Cm-S6k*, suggesting that up-regulation of mTOR signaling is not necessary for YO ecdysteroidogenesis.

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