INTRODUCTION

Hypoxia, defined as dissolved oxygen (DO) level that falls below 2.8 mg O$_2$ l$^{-1}$, affects several 100 000 km$^2$ of marine waters worldwide (UNEP 2011). Previous studies have shown that hypoxia results in aberrant behaviours of benthic fauna, decline of fisheries, and major changes in the structure and trophodynamics of marine ecosystems (Wu 2002, Díaz & Rosenberg 2008, Liu et al. 2011a,b). Although hypoxia is a naturally occurring phenomenon, increasingly it is caused by eutrophication of coastal waters due to anthropogenic activities, especially sewage discharge and the wide use of agricultural fertilizers (Gilbert et al. 2010, Rabalais et al. 2010). Studies show that the number of hypoxic areas has doubled in each decade since the 1960s (Díaz & Rosenberg 2008, 2011, Diaz & Rabalais 2009, Levin et al. 2009, Rabalais et al. 2010). Recently, the United Nations Environment Programme (UNEP) predicted that the largest increase in the number of hypoxic areas in the coming decade would occur in Asia (UNEP 2011).

Previous studies have collectively suggested that marine organisms attempt to maintain oxygen delivery and conserve energy during hypoxia by increasing respiration rate, decreasing metabolic rate, down-regulating protein synthesis, or modifying certain regulatory enzymes (Wu 2002, 2009). Eventually, these responses and the resulting physiological adjustments may manifest themselves in growth reduction. Growth reduction has been previously demonstrated in adults of the polychaete *Capitella*...
sp. (Forbes & Lopez 1990), green sea urchin *Strongylocentrotus droebachiensis* (Siikavuopio et al. 2007), and gastropod *Nassarius festivus* (Cheung et al. 2008). A similar effect on growth rate was observed in juveniles of the abalone *Haliotis laevigata* (Harris et al. 1999), blue crabs *Callinectes similis* and *C. sapidus* (Das & Stickle 1993), Chinese shrimp *Fenneropenaeus chinensis* (Wei et al. 2008), amphipod *Melita longidactyla* (Wu & Or 2005), American oyster *Crassostrea virginica* (Baker & Mann 1992), and green-lipped mussel *Perna viridis* (Wang et al. 2012) as well as larvae of the copepod *Acartia tonsa* (Richmond et al. 2006), blue mussel *Mytilus edulis* (Wang & Widdows 1991), and gastropod *N. festivus* (Chan et al. 2008).

Over the past decade or so, emerging evidence shows that early development is the most sensitive stage and that early experience is a strong predictor of phenotype across the life history. Latent effects, also called carryover effects, are defined as the effects resulting from conditions experienced in early development, such as the larval stage, that are expressed in later life, such as the juvenile stage (Marshall et al. 2003, Pechenik 2006). The latent effects of nutrition, delayed metamorphosis, and physical factors (including temperature and salinity stress, increased energy expenditure, low pH, and UV irradiation) as well as chemical factors (i.e. heavy metals) have been previously demonstrated in marine invertebrates, fish, amphibians, and insects (reviewed by Pechenik 2006; also see Cebrian & Uriz 2007, Thiyagarajan et al. 2007, Kurihara 2008, Villanueva et al. 2008, Parker et al. 2009, 2012, Onitsuka et al. 2010, Diederich et al. 2011, Martin et al. 2011). For instance, limited food or short-term starvation during the larval stage of the gastropod *Crepidula onyx* could significantly reduce the juvenile growth rate (Chiu et al. 2007, 2008a).

The exposure of organisms to hypoxia during their early development may result in immediate effects, such as mortality and growth rate reduction, and effects that persist across the life history—the latent effects, which, if present, may have major ecological consequences over the coastal areas that are affected by hypoxia. The marine benthic gastropod *Crepidula onyx* is found in great abundance in intertidal and subtidal waters along the Pacific coast of North and South America, China, Japan, and Hong Kong (Plutchak et al. 2006). Using *C. onyx* as a model invertebrate species, we tested the hypothesis that exposure to hypoxia throughout the larval stage has effects that persist to the juvenile stage and significantly reduce the juvenile growth rate. *C. onyx* is found in Victoria Harbour, Hong Kong. The monitoring data of the Environmental Protection Department Hong Kong (EPD) between 2003 and 2007 revealed that the bottom waters of Victoria Harbour were affected by hypoxia and that the DO was <3 mg O2 l−1 for ~10% of the summer (EPD 2010). Finally, periodic hypoxic events are caused by over-supply of nutrients, which promotes phytoplankton growth. Therefore, during hypoxia, larvae may experience variable concentrations of microalgae, which are their food source. In this study, we investigated and compared the latent effects of hypoxia under 2 different algal food concentrations.

**MATERIALS AND METHODS**

**Study organism**

Adults of *Crepidula onyx* were collected from the low intertidal zone in Victoria Harbour, located north of Hong Kong Island, Hong Kong (22° 17' N, 114° 10' E), and acclimatized in the laboratory for 1 mo on a diet of the naked flagellate *Isochrysis galbana* (Tahitian strain, Clone T-ISO) (Chiu et al. 2008b). The adults usually brood once every 4 wk. Egg capsules were located underneath the female foot. After brooding for about 2 wk, thousands of free-swimming, veliger larvae were released and used for the experiments as described below. The *C. onyx* larvae are fully planktotrophic. A concentration of 1 × 10^4 cells l−1 of *I. galbana* was the minimum required to sustain growth and development in laboratory culture but at a rate significantly slower than that of 1.8 × 10^5 cells l−1 (Zhao et al. 2003). In addition, when provided with the latter food concentration and maintained at 24°C, the larvae became physiologically competent to metamorphose into juveniles after 5 to 7 d (Zhao et al. 2003) or 8 d (Chiu et al. 2012).

**Hypoxia exposure**

The gas-mixing tanks, each of which supplied 3 experimental chambers for each treatment, were injected with nitrogen gas and air, following the design described in our previous study with slight modifications (Fig. 1) (Liu et al. 2011b). The flow of nitrogen gas and air was regulated by a DO controller (Cole-Parmer 01972-00) connected to electromagnetic valves that opened or closed depending on the DO level in the experimental chambers, as monitored using an optical DO meter (TauTheta SOO-100). Seawater for the normoxic control was bubbled with air only.
Experimental design

The hypoxic effects on mortality were examined at the 4 DO levels 1, 2, 3, and 6 mg O$_2$ l$^{-1}$, while the effects of hypoxia on larval growth and development and the subsequent latent effects on juvenile growth rate were examined at the 3 DO levels 2, 3, and 6 mg O$_2$ l$^{-1}$, corresponding to the stronger hypoxic treatment, weaker hypoxic treatment, and normoxic control, respectively. The latter experiment was carried out under separate high and low food concentrations. Immediately after hatching, the larvae were maintained on a diet of live *Isochrysis galbana* at $2 \times 10^5$ cells l$^{-1}$ for the high food experiment or at half of this concentration (i.e. $1 \times 10^5$ cells l$^{-1}$) for the low food experiment.

All treatments and replicates for each of the 3 experiments (i.e. Expt 1: range finding test, Expt 2: high food experiment, and Expt 3: low food experiment) were carried out at the same time. Depending on the number of levels of DO tested (4 levels for Expt 1 and 3 for Expts 2 and 3), each experiment had 12 or 9 experimental chambers (i.e. glass beakers with a capacity of 250 or 1200 ml). Each DO level had 3 replicate chambers, and each chamber consisted of 200 larvae and 200 ml seawater for Expt 1 or 1000 larvae and 1000 ml seawater for Expts 2 and 3 (i.e. 1 larva ml$^{-1}$).

Newly hatched larvae were exposed to the hypoxic treatments and control for 8 or 10 d (i.e. all larvae either settled onto the Petri dishes [Falcon no. 1006] provided or died after 8 or 10 d post-hatching). Seawater with a salinity of 30 psu was used and maintained at 24°C. Also, half of the seawater in the chambers was changed daily. New seawater was adjusted for the desired DO level prior to changing. Furthermore, all larvae were examined every day (the larvae exposed to 1 mg O$_2$ l$^{-1}$ tended to be a little sluggish), and dead larvae were removed from the experimental chambers. Cumulative mortality was determined for the whole larval stage.

For the high and low food experiments, 10 individuals were sampled from each replicate chamber once daily (without replacement), and their shell lengths were measured to the nearest 1 µm using a direct light microscope equipped with an ocular micrometer at 100×. Individuals were used in total lipid content analysis and settlement bioassays when the majority became morphologically competent (i.e. 8 d old larvae for all treatments in the high food experiment and for the normoxic control in the low food experiment; 10 d old larvae for the hypoxic treatments in the low food experiment). Groups of competent larvae of 20 individuals each were rinsed with distilled water, lysed by ultrasonic pulses (Branson Sonifier 450) for 2 min, and subsequently extracted for total lipids. Total lipid content was quantified by sulfuric acid charring (Mann & Gallager 1985), with tripalmitin as the standard. The morphological competence was assessed by checking for the occurrence of shell brims (i.e. larvae with shell brims resemble brimmed hats, indicating the transformation of the geometry of shell growth from the spiral pattern characteristic of pre-competent larvae to the linear form characteristic of adults) (Pechenik 1980). Settlement assays were carried out following the procedures developed in our previous study (Chiu et al. 2008b). Briefly, for each replicate chamber, 30 larvae were pipetted into a Petri dish (Falcon No. 1006) and induced to settle in 10 ml of still seawater whose K$^+$ concentration had been elevated by 15 mM. After 6 h, the numbers of larvae that successfully settled (as indicated by a significant reduction in size of the velar lobes) were counted (Chiu et al. 2007). Metamorphosis is easy to observe in *Crepidula onyx*, as in other calyptraeids with planktotrophic larvae (Henry et al. 2010). Besides elevated K$^+$ concentration, metamorphosis of *C. onyx* larvae can also be induced by exposure to adult-conditioned seawater (Zhao & Qian 2002) and biofilms (Chiu et al. 2008b).
Latent effects

For each replicate chamber in the high and low food experiments, 12 competent larvae were pipetted into 1 of 10 Petri dishes (Falcon No. 1006) and induced to settle in 10 ml of still seawater whose $K^+$ concentration had been elevated by 15 mM. In all of the treatments, the mean percent settlement was >80%, so there were >10 newly settled larvae in each dish. Larvae that did not settle after 6 h were discarded. Newly settled larvae were then transplanted to Victoria Harbour and allowed to grow for 14 d. Previous study demonstrated latent effects of starvation on growth of Crepidula onyx juveniles in a 12 d outplant experiment (Chiu et al. 2008a). In this study, the high food experiment was conducted in November 2011, and water temperature, salinity, and chl $a$ concentration were 25.6°C, 32.5 psu, and 0.5 µg l$^{-1}$, respectively; the low food experiment was conducted in December 2011, and water temperature, salinity, and chl $a$ concentration were 21.6°C, 32 psu, and 0.9 µg l$^{-1}$, respectively (EDP 2011). The Petri dishes from different replicates of the same DO treatment were mounted on the same plastic frame (50 cm length × 55 cm width), put in a plastic mesh box (27.5 cm length × 19 cm width × 22 cm depth) with a mesh size of 4 mm (to shelter the newly settled individuals from relatively large fish predators), and then deployed at the low intertidal zone. In this study area, tidal oscillation was semidiurnal, with a mean range of 1.6 m during the 2 outplant periods. Plastic frames of different DO treatments were put at the same tidal height, 1 to 2 m apart (the frame locations were randomized). Some juveniles died or moved away from the substrata. The rate of mortality and emigration in the high food experiment ranged from 21.5 and 38% in the 2 and 3 mg O$_2$ l$^{-1}$ treatments to 62% in the 6 mg O$_2$ l$^{-1}$ treatment, while the rate in the low food experiment was 13.3, 6.3, and 6.7% in the 2, 3, and 6 mg O$_2$ l$^{-1}$ treatments, respectively. The remaining juveniles were brought back to the laboratory. A group of ca. 50 juveniles was placed in 500 ml of Isochrysis galbana suspension at a concentration of 2 × 10$^4$ cells l$^{-1}$ in each of the 3 replicate beakers and maintained in the dark at 24°C for 5 h. The algal concentration was measured at hourly intervals, and control consisted of beakers containing only algae (i.e. no juvenile). Filtration rate was calculated following the equation $V = \text{volume of water, } n = \text{number of larvae, } t = \text{time, } C_0 = \text{control concentration, and } C_1 = \text{experimental concentration (Beiras & Camacho 1994).}$

Statistical analysis

The data were checked by the Shapiro-Wilk normality test and equal variance test (Zar 1999). Percentages of cumulative mortality and settlement were arcsine transformed before analysis (Zar 1999). The data obtained from individuals in a replicate (i.e. experimental chamber) were first combined and averaged for one mean from that replicate. A 1-way ANOVA was used to test the difference in cumulative mortality, shell length, percent settlement, total lipid content, growth rate, dry weight, and filtration rate. If a significant difference was found by ANOVA, Tukey’s multiple comparisons were used to identify the differences between the various treatments. Data were significantly different at $\alpha = 0.05$.

RESULTS

Expt 1: range finding test

The cumulative mortality of Crepidula onyx was significantly affected by the DO treatment ($F_{3,8} = 17.78, p < 0.001$) (Fig. 2). The majority of the larvae in the strongest hypoxic treatment (i.e. 1 mg O$_2$ l$^{-1}$) died after 8 d post-hatching, in contrast to the weaker hypoxic treatments (i.e. 2 and 3 mg O$_2$ l$^{-1}$) and normoxic control (i.e. 6 mg O$_2$ l$^{-1}$). The median lethal temperature (LT$_{50}$) for 1 mg O$_2$ l$^{-1}$ was 3.5 d. Moreover, the means of the cumulative mortality of the weaker hypoxic treatments and normoxic control were not statistically different; the DO levels of 2 and 3 mg O$_2$ l$^{-1}$ were, hence, sub-lethal for the C. onyx larvae.

Expt 2: high food experiment

The 8 d old larvae grew to a mean (±SD) size ranging from 682 (±47) to 727 (±31) µm (i.e. in shell concentration were 25.6°C, 32.5 psu, and 0.5 µg l$^{-1}$, re-
length) (Fig. 3A), and the means of different treatments and control were not statistically different ($F_{2,6} = 3.346, p = 0.106$). The mean total lipid content (± SD) of the larvae was 7.6 (± 0.6), 8.6 (± 1.0), and 9.3 (± 0.5) µg ind.$^{-1}$ for the 2, 3, and 6 mg O$_2$ l$^{-1}$ treatments, respectively, and were not statistically different ($F_{2,6} = 3.384, p = 0.104$). The larvae also reached metamorphic competency, and >80% settled onto the substrates provided (Petri dishes, Falcon No. 1006) (Fig. 3B). Larvae that did not metamorphose showed reduced movement and gradually died on the following days. There was no significant effect of the DO treatment on the percent settlement ($F_{2,6} = 1.607, p = 0.276$).

The mean growth rate of the juveniles (i.e. that were developed from the larvae that had received different DO treatments in the laboratory and subsequently transferred to the field) ranged from 80.0 (± 2.5) to 87.6 (± 4.6) µm d$^{-1}$ (Fig. 4A). After 2 wk post-settlement, these juveniles attained a mean dry...
weight of 280 (±30) to 313 (±85) µg ind.\(^{-1}\) (Fig. 4B) and a mean filtration rate of 225 (±67) to 342 (±18) µl h\(^{-1}\) ind.\(^{-1}\) (Fig. 4C). There was no significant effect of the DO treatment on the growth rate \((F_{2,6} = 4.124, p = 0.075)\), dry weight \((F_{2,6} = 0.234, p = 0.798)\), and filtration rate \((F_{2,6} = 1.462, p = 0.304)\).

**Expt 3: low food experiment**

Significant differences in the shell length \((F_{2,6} = 17.39, p = 0.003)\) and percent settlement \((F_{2,6} = 9.302, p = 0.015)\) of the 8 d old larvae were evident among DO treatments. The 8 d old larvae in the 6 mg O\(_2\) l\(^{-1}\) treatment had a mean (± SD) shell length of 630 (±42) µm, which was significantly larger than 542 (±50) µm in the 2 mg O\(_2\) l\(^{-1}\) treatment (Fig. 5A). Also, 85% of the larvae from the 6 mg O\(_2\) l\(^{-1}\) treatment attained metamorphic competency by 8 d and settled onto the substrates provided (Fig. 5B). The larvae in the hypoxic treatments reached metamorphic competency later (83.3 ± 5.8% and 96.7 ± 5.8% in the 2 and 3 mg O\(_2\) l\(^{-1}\) treatments, respectively) and at 10 d had a mean shell length of 614 to 638 µm (Fig. 5C,D). No significant difference was observed for the shell length \((F_{1,4} = 2.592, p = 0.183)\) and percent settlement \((F_{1,4} = 6.954, p = 0.058)\) between the 2 hypoxic treatments at 10 d post-settlement. Nevertheless, the mean shell length of the 8 d old larvae from the normoxic control and the 10 d old larvae from the 2 hypoxic treatments (i.e. during settlement) did not differ significantly \((F_{2,6} = 1.132, p = 0.383)\). These competent larvae also had a mean total lipid content (±SD) of 9.1 (±0.9), 8.9 (±1.9), and 9.7 (±0.6) µg ind.\(^{-1}\) for the 2, 3, and 6 mg O\(_2\) l\(^{-1}\) treatments, respectively. Similarly, these means did not differ significantly \((F_{2,6} = 0.382, p = 0.732)\).

The juvenile growth rate, dry weight, and filtration rate were significantly affected by the DO treatment (Fig. 6). When compared with the normoxic control, the 2 hypoxic treatments had a 9.5 to 13.8% reduced growth rate (Fig. 6A), 58% reduced dry weight (Fig. 6B), and 26.5 to 30.8% reduced filtration rate (Fig. 6C).

**DISCUSSION**

The latent effects of hypoxia exposure during the larval stage on the juvenile growth rate were evident for *Crepidula onyx* only when the larvae were fed at a low food concentration. When compared with the normoxic control, the 2 and 3 mg O\(_2\) l\(^{-1}\) treatments had ca. 14 and 10% reduced juvenile growth rate, respectively. However, the latent effects were only observed when the larvae were fed with *Isochrysis galbana* at 1 × 10\(^5\) cells l\(^{-1}\), a low food concentration (Fig. 6), but not at the doubled food concentration (Fig. 4). The latent effects may be explained by 2 different mechanisms with different predictions: (1) depletion of energy reserves (i.e. small larval size and low energy reserve at metamorphosis) and (2) small size of the juvenile feeding apparatus, which in turn decreases the animal’s ability to feed (i.e. low filtration and, hence, feeding rates).

The depletion of energy reserves mechanism assumes that phenotypic traits such as the size and energy reserve at metamorphosis (often as a result of a combination of larval experience and initial plasticity and heterogeneity among larvae of same brood) correlate with the juvenile and adult fitness (Marshall & Keough 2006, Pechenik 2006, Van Allen et al. 2010, Dupont et al. 2012). For example, Emlet &
Sadro (2006) demonstrated that a reduction in larval food ration resulted in cyprids of smaller size (with less lipid) and, subsequently, reduced juvenile growth rate in the barnacle *Balanus glandula*. Similar results were found for the effects of delayed metamorphosis on cyprids (smaller size and with less lipid) and juvenile growth rate (reduced) in the barnacle *B. amphitrite* (Thiyagarajan et al. 2007). Nevertheless, Emlet & Sadro (2006) and Thiyagarajan et al. (2007) suggested that higher food availability to barnacle juveniles cannot make up for the deleterious effects of larval experience (i.e. poor nutrition and delayed metamorphosis) on reduced juvenile growth rate.

A correlation was observed between increased swimming activity of the lecithotrophic larvae (i.e. they do not feed as opposed to *Crepidula onyx* larvae), reduced energy content, and reduced colony growth in the colonial ascidian *Diplosoma listerianum* (Marshall et al. 2003) and bryozoan *Bugula neritina* (Wendt 1998). Furthermore, the effects of delayed metamorphosis for *B. neritina* were offset by larval access to dissolved organic matter, suggesting a link between larval energy reserves and latent effects (Wendt & Johnson 2006). Nonetheless, there was a qualitative mismatch between the effects of larval starvation on competent larvae (effects on size and lipid content) versus juvenile growth for the Mediterranean mussel *Mytilus galloprovincialis* (Phillips 2004). In our study, the *C. onyx* larvae from the hypoxic treatments settled and metamorphosed at a size and total lipid content similar to the normoxic control larvae (but not of the same age). The depletion of energy reserves mechanism does not seem able to explain the observed effects of hypoxia on juvenile growth rate.

Our data indicate a reduction in filtration rate of the juveniles from the hypoxic treatments at 2 wk after settlement in the low food experiment (Fig. 6C). The results here add to the growing evidence (e.g. Pechenik 2006, Chiu et al. 2007, 2008a) that the latent effects may be at least partially due to a low filtration rate. Hypoxic stress during early development may interfere with processes associated with juvenile gill formation, causing a low filtration rate and, hence, growth rate reduction. It is not clear how exposure to hypoxia during early development may lead to reduced filtration rate, yet this reflects smaller or abnormal juvenile gill structure or function. It has been suggested that early experience might interfere with the timing and magnitude of transcriptional processes, some of which may be associated with gill development (Pechenik 2006, Williams & Degnan 2009).

While the larvae of *Crepidula onyx* exposed to 1 mg O₂ l⁻¹ died before they could become competent to metamorphose (i.e. the LT₅₀ at 1 mg O₂ l⁻¹ was 3.5 d) (Fig. 2), they survived mild hypoxia (i.e. DO ≥ 2 mg O₂ l⁻¹) and grew and developed into competent larvae under this condition (Figs. 3 & 5). Previous studies reported the lethal effects of hypoxia on marine invertebrates, for example, polychaetes, bivalves, crabs, and gastropods (reviewed by Gray et al. 2002; also see Chan et al. 2008, Bell et al. 2010, Liu et al. 2011a,b). The finding here is consistent with previous studies on mollusk larvae, including the blue mussel *Mytilus edulis* (Wang & Widdows 1991), the short-
necked clam *Ruditapes philippinarum* (Toba et al. 2008), and the gastropods *Nassarius festivus* (Chan et al. 2008), *N. siquijorensis*, and *N. conoidalis* (Liu et al. 2011a)—the lethal DO level is ≤2 mg O₂ l⁻¹ for these larval species and for *C. onyx* larvae.

Under the low food concentration, both hypoxic treatments (2 and 3 mg O₂ l⁻¹) increased the time needed for the larvae to be competent to metamorphose, but there was no discernible effect on larvae or juveniles when the food concentration was doubled. Previous studies have suggested that marine molluscs attempt to maintain oxygen delivery during hypoxia by avoidance behaviour (for example, an increased dispersal velocity of the *Nassarius conoidalis* larvae: Liu et al. 2011a; and a significant upward swimming movement of the *M. edulis* larvae: Wang & Widdows 1991) and/or increasing ventilation rate (for example, the oyster *Crassostrea virginica*: Ivanina et al. 2011; and the quahog *Arctica islandica*: Strahl et al. 2011). These responses to hypoxia are energetically costly and may cause a reduction in growth rate (Wu 2002, 2009). Our results here suggest that high food concentration may offset the effects of hypoxia on reduced growth rate, possibly by providing the organisms with extra energy to perform these responses to maintain oxygen delivery.

Hypoxia is a global environmental problem, threatening the ecosystem health of several 100,000 km² of marine waters worldwide (UNEP 2011). There have been numerous studies investigating the effects of hypoxia on the marine organisms (for example, Forbes & Lopez 1990, Diaz & Rosenberg 1995, Harris et al. 1999, Wu & Or 2005, Chan et al. 2008, Cheung et al. 2008, Brante et al. 2009). The present study, however, was the first to provide evidence of the effects of hypoxia exposure during an early developmental stage on the downstream life stage.

**Acknowledgements.** The manuscript benefited greatly from the comments and suggestions of 3 anonymous reviewers, to whom we are grateful. We also thank Dr. Rajan for helpful comments. The work described in this paper was fully supported by a grant from the Research Grants Council of the Hong Kong Special Administrative Region, China (AoE/P-04/04).

**LITERATURE CITED**


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EPD (2011) Annual marine water quality reports 2011. Environmental Protection Department, Hong Kong SAR Government, Hong Kong


Li & Chiu: Latent effects of hypoxia on marine invertebrates

Li CC, Chiu JMY, Li L, Shin PKS, Cheung SG (2011b) Physiological responses of two sublittoral nassariid gastropods under reduced oxygen levels: implications for their distributions in Hong Kong waters. Mar Pollut Bull 63:230–236


Editorial responsibility: Hans Heinrich Janssen, Oldendorf/Luhe, Germany

Submitted: May 2, 2012, Accepted: December 3, 2012
Proofs received from author(s): March 21, 2013